BARD Final Report IS-4784-14

Waste to Worth: Active antimicrobial and health-beneficial food coating from byproducts of mushroom industry

Poverenov, E. Agricultural Research Organization
McHugh, T. USDA, Agricultural Research Service
Rodov, V. Agricultural Research Organization

Project award year: 2014
Three year research project

Abstract

Background. In this proposal we suggest developing a common solution for three seemingly unrelated acute problems: (1) improving sustainability of fast-growing mushroom industry producing worldwide millions of tons of underutilized leftovers; (2) alleviating the epidemic of vitamin D deficiency adversely affecting the public health in both countries and in other regions; (3) reducing spoilage of perishable fruit and vegetable products leading to food wastage. Based on our previous experience we propose utilizing appropriately processed mushroom byproducts as a source of two valuable bioactive materials: antimicrobial and wholesome polysaccharide chitosan and health-strengthening nutrient ergocalciferol (vitamin D_2). Additional benefit of these materials is their origin from non-animal food-grade source. We proposed using chitosan and vitamin D as ingredients in active edible coatings on two model foods: highly perishable fresh-cut melon and less perishable health bars.

Objectives and work program. The general aim of the project is improving storability, safety and health value of foods by developing and applying a novel active edible coating based on utilization of mushroom industry leftovers. The work plan includes the following tasks: (a) optimizing the UV-B treatment of mushroom leftover stalks to enrich them with vitamin D without compromising chitosan quality - Done; (b) developing effective extraction procedures to yield chitosan and vitamin D from the stalks - Done; (c) utilizing LbL approach to prepare fungal chitosan-based edible coatings with optimal properties - Done; (d) enrichment of the coating matrix with fungal vitamin D utilizing molecular encapsulation and nano-encapsulation approaches - Done, it was found that no encapsulation methods are needed to enrich chitosan matrix with vitamin D; (e) testing the performance of the coating for controlling spoilage of fresh cut melons - Done; (f) testing the performance of the coating for nutritional enhancement and quality preservation of heath bars - Done. Achievements. In this study numerous results were achieved. Mushroom waste, leftover stalks, was treated with UV-B light and treatment induces a very high accumulation of vitamin D_2, far exceeding any other dietary vitamin D source. The straightforward vitamin D extraction procedure and a simplified analytical protocol for time-efficient determination of the vitamin D_2 content suitable for routine product quality control were developed. Concerning the fungal chitosan extraction, new freeze-thawing protocol was developed, tested on three different mushroom sources and compared to the classic protocol. The new protocol resulted in up to 2-fold increase in the obtained chitosan yield, up to 3-fold increase in its deacetylation degree, high whitening index and good antimicrobial activity. The fungal chitosan films enriched with Vitamin D were prepared and compared to the films based on animal origin chitosan demonstrating similar density, porosity and water vapor permeability. Layer-by-layer chitosan-alginate electrostatic deposition was used to coat fruit bars. The coatings helped to preserve the quality and increase the shelf-life of fruit bars, delaying degradation of ascorbic acid and antioxidant capacity loss as well as reducing bar softening. Microbiological analyses also showed a delay in yeast and fungal growth when compared with single layer coatings of fungal or animal chitosan or alginate. Edible coatings were also applied on fresh-cut melons and provided significant improvement of physiological quality (firmness, weight loss), microbial safety (bacteria, mold, yeast), normal respiration process (CO_2, O2) and did not cause off-flavor (EtOH). It was also found that the performance of edible coating from fungal stalk leftovers does not concede to the chitosan coatings sourced from animal or good quality mushrooms. Implications. The proposal helped attaining triple benefit: valorization of mushroom industry byproducts; improving public health by fortification of food products with vitamin D from natural non-animal source; and reducing food wastage by using shelf-life-extending antimicrobial edible coatings. New observations with scientific impact were found. The program resulted in 5 research papers. Several effective and straightforward procedures that can be adopted by mushroom growers and food industries were developed.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Reviewed	0	1	0
Submitted	0	3	0

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
Postdoctoral Fellow	Ban	bzhaojun	Agricultural research organization	Israel
Ph.D. Student	Shlar	Ilia	Agricultural research organization	Israel
Postdoctoral Fellow	Sedej	Ivana	USDA	USA
Postdoctoral Fellow	Bilbao Sainz	Cristina technitian	USDA	Israel
Ph.D. Student	Rutenberg	Roi	ARO	Israel
M.Sc. Student	Rosenthal	Ingrid	USDA-ARS	USA
Ph.D. Student	Arnon-Ripps	Hadar	ARO	Israel
M.Sc. Student	Punotai	Kaylin	USDA	USA

Contribution of the collaboration

a) IS team provided protocols for production of chitosan and vitamin D from the mushroom. Both teams, US and IS teams utilized these protocols for further studies.
b) US team provided mushroom powder treated by UV-B irradiation utilizing different conditions and IS team is determining the effect of UV treatment on vitamin D and chitosan content. This work allows determining of the optimal irradiation protocol by US team. The protocol was utilized for further studies.
c) IS team developed an advanced protocol (freeze-thawing approach) for efficient yielding of fungal chitosan. The physicochemical and antimicrobial properties of the prepared chitosan were studied. The protocol was used in the further studies.
d) Mushroom samples treated by various UV doses were produced by US team and sent to the IS team.
e) Vitamin D content of mushrooms samples after different UV-treatments was studied and quantified by IS team.
f) The edible films based on chitosan and vitamin D enriched mushroom powder were prepared and their chemical, physical and mechanical properties were studied by US team.
g) Edible coatings formulation were formed and applied on fruit bar (US team) and fresh-cut melon (IS team) and their effect on food quality, microbial safety and storability was studied.

The new methodologies developed in one team were demonstrated to the partner from other team during their visit to US/Israel. These methodologies have already been implemented and will be implemented in further activities of both laboratories especially in the interaction with the mushroom industries.

The research program resulted in writing of 5 mutual papers. General discussion of the findings and experiences of the project brought the partners to the understanding that wider international multilateral collaboration and interaction are needed in the area of agrifood waste valorization, in particular between USA and Israeli researchers, as well as European and Asian colleagues. A decision was taken to organize a workshop (BARD-supported) continuing the line of this project.

Major Achievements

Vitamin D

Application of UV-B light. Irradiation of cut-off stipe bases of common mushrooms (*Agaricus bisporus*) with UV dose of 1.0 J cm^{-2} resulted in vitamin accumulation of about 80 µg g^{-1} for a white strain and about 100 µg g^{-1} for the brown one. In comparison, untreated mushrooms have vitamin D_2 concentrations of 0.0003-0.0008 µg/g. Although there it is known that higher concentration of ergosterol (vitamin D2 precursor) are in the caps, the vitamin D_2 content found in one gram of treated mushroom stalks is still 4.5 to 6 times the RDA for vitamin D recently established by the Food and Nutrition Board of the Institute of Medicine. This indicated the effectiveness of UV-B irradiation for increasing vitamin D_2 content in mushrooms (Figure 1).

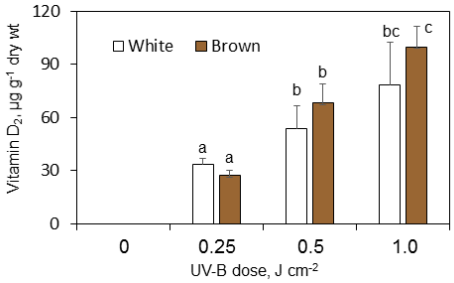

Figure 1. Effect of UV-B dose on the content of vitamin D_2 in the stipe cut-offs of white and brown mushrooms. Values represent means of three replications; error bars 95% *t* confidence intervals.

Vitamin D quantification. The existing procedures for vitamin D quantification were developed for fat-rich foods with relatively low vitamin content, e.g. oily fish. In contrast, mushrooms are low in fat and upon UV irradiation accumulate high levels vitamin D_2 (ergocalciferol). A simplified analytical procedure for measuring the content of vitamin D_2 in UV-treated mushrooms was developed. The procedure omits time- and labor-consuming stages of saponification and preparative enrichment and is based on direct extraction from ground dry material with subsequent reverse-phase HPLC. The procedure is suitable for the analysis of samples containing above 1 µg/g of vitamin D_2, far below the vitamin content reported in UV-treated mushrooms.

An advanced protocol for yielding good quality fungal chitosan

Fungal chitosan was produced from brown *Agaricus bisporus*, white *Agaricus bisporus*, and *Pleurotus ostreatus* mushrooms by utilizing a classic alkali deacetylation protocol (PI) and a new protocol (PII) that involves a freeze-thawing cycle. The new protocol, PII led to an up to 2-fold increase in the chitosan yield, a more efficient deacetylation process, up to 3-fold decrease of acetyl units and whiter color (Table 1).

Table 1. Comparison of Yield, Whiteness index and Degree of acetylation (% DA) for the three mushroom sourced chitosans produced by either Protocol I and Protocol II.

	brown *A. bisporus*		
	Yield (mg g^{-1})	Whiteness index	DA (%)
Protocol I	31.8±4.2b	77.40±1.16a	35.89±5.39b
Protocol II	42.3±0.7a	80.04±1.62a	17.24±1.44a
	white *A. bisporus*		
	Yield (mg g^{-1})	Whiteness index	DA (%)
Protocol I	33.3±2.1b	74.18±2.15a	48.13±4.23b
Protocol II	37.7±1.8a	77.38±0.52a	27.71±3.69a
	Pleurotus ostreatus		
	Yield (mg g^{-1})	Whiteness index	DA (%)
Protocol I	24.4±4.5b	75.67±0.10b	44.11±4.27b
Protocol II	44.3±3.4a	80.28±0.73a	14.84±1.55a

Gel permeation chromatography, FTIR, NMR, UV, elemental analysis, X-ray diffractometry, and thermogravimetric analysis were all used to characterize the prepared fungal chitosan samples and examine their physical properties such as viscosity, color, hydrophobicity and solubility (Table 2).

Table 2. Average molecular weights (Mw) [Da], molecular weight average numbers (Mn) [Da], peak molecular weights (Mp) [Da], and polydispersity indices (PDI) for all the inspected chitosan samples.

Chitosan source	Mw/ (Da)	Mn/ (Da)	Mp/ (Da)	PDI Mw/Mn	[η] (mL g^{-1})	Water contact angle (°)	Solubility (g L^{-1})
crustacean-sourced	59,826	16,353	31,859	3.66	454	102.38 ± 1.47	9.06 ± 0.930a
brown *A. bisporus*	600,858	96,937	262,982	6.2	406	84.73 ± 1.28	9.45 ± 1.46a
white *A. bisporus*	55,645	24,342	31,206	2.29	343	101.55 ± 3.88	7.51 ± 0.60b
P. ostreatus	24,306	11,387	10,649	2.13	232	*	8.82 ± 0.54ab

Fungal chitosan activity was tested on *E. coli* bacteria and *Saccharomyces cerevisiae* yeast and found to be similar to that of animal-sourced commercial chitosan (Figure 2).

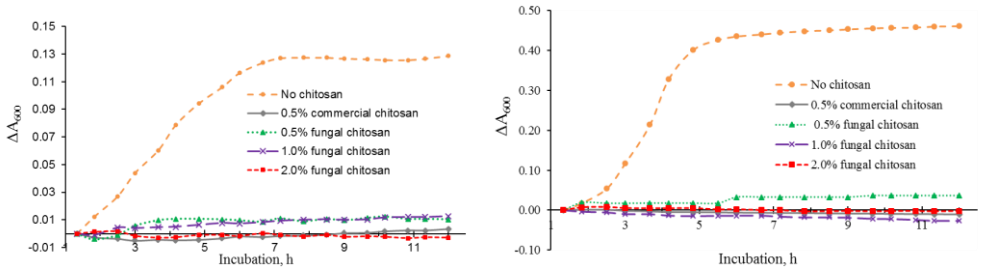

Figure 2. Antimicrobial activity on *E. coli* bacteria (left) and *Saccharomyces cerevisiae* yeast (right)

Chitosan films enriched with vitamin D

The freeze-dried mushroom powder retained its vitamin D_2 and was further used as an ingredient to prepare nutritionally fortified chitosan films and coatings. Four types of films were cast based on fungal chitosan, fungal chitosan containing vitamin D enriched mushroom particles, high molecular weight (HW) and low molecular weight (LW) commercial chitosan from animal origin. The fungal chitosan films showed

similar density, porosity and water vapor barrier properties to animal-sourced chitosan. However, fungal chitosan films were more hydrophobic and less flexible than the LW and HW chitosan films. Addition of mushroom particles did not significantly affect mechanical or permeability properties of the films.

Chitosan-alginate Layer-by-layer edible coatings of fruit bars

Layer-by-layer (LbL) electrostatic deposition of the polycation chitosan and the polyanion alginate was used to coat fruit bars enriched with ascorbic acid. The performance of the LbL coatings was compared with those containing single layers of fungal chitosan, animal origin chitosan and alginate. LbL coated bars showed highest ascorbic acid content, antioxidant capacity, firmness and fungal growth prevention.

Edible coatings of fresh-cut melon

The chitosan edible coatings from two sources fungal stipe offcuts and cap were applied on fresh-cut melons. The stipe source resulted in higher yield of chitosan than cap. As edible coating, fungal chitosan from both stipe and cap significantly inhibited physiological and microbial deterioration of melon, thereby reducing the bacteria, yeast and mold counts (Table 3) and slowing down tissue texture degradation.

Table 3. Total aerobic counts and total mold and yeast counts (log CFU g^{-1}) of fresh-cut melons during storage at 6 °C. Values represent means of nine replications ± 95%.

PCA (log CFU/gr)	**0 days**	**5 days**	**9 days**	**11 days**
fungal chitosan- cap	1.95 ±0.14^{a}	4.05 ±0.29^{bc}	6.39 ± 0.40^{b}	6.92± 0.45^{b}
fungal chitosan- stipe	1.94 ± 0.12^{a}	6.33 ± 0.40^{ab}	6.44 ± 0.40^{b}	6.54 ± 0.23^{b}
control	2.00 ± 0.24^{a}	7.98± 0.45^{a}	8.95± 0.32^{a}	9.04± 0.20^{a}
commercial chitosan	2.00 ±0.07^{a}	5.40 ± 0.45^{b}	6.85 ± 0.32^{b}	7.00 ± 0.32^{b}
PDA+A (log CFU/gr)	**0 days**	**5 days**	**9 days**	**11 days**
fungal chitosan- cap	0.69 ± 0.10^{a}	0.91 ± 0.11^{b}	1.22 ± 0.11^{c}	3.70 ± 0.05^{b}
fungal chitosan- stipe	0.8 ± 0.05^{a}	1.00 ± 0.13^{b}	1.90 ± 0.14^{c}	3.20 ± 0.20^{b}
control	0.8 ± 0.12^{a}	1.90 ± 0.20^{a}	4.12 ± 0.20^{a}	4.21± 0.20^{a}
commercial chitosan	0.70 ± 0.09^{a}	0.89 ± 0.08^{b}	3.26 ± 0.26^{b}	3.67 ± 0.15^{b}

Agricultural and economic impact

The proposal helped attaining triple benefit: valorization of mushroom industry byproducts; improving public health by fortification of food products with vitamin D from natural non-animal source; and reducing food wastage by using shelf-life-extending antimicrobial edible coatings. Several effective and straightforward procedures that can be adopted by mushroom growers and food industries were developed: simplified vitamin D analysis, freeze-thaw protocol for chitosan yield, successful application of active edible coatings to improve food quality and storability.

Changes in work plan

The US team visit took place at the third year instead of the end of second year as was planned. We received BARD permission for this change.

Publications for Project IS-4784-14

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Submitted	Reviewed	*Z. Ban, B. Horev, Rץ Rutenbergת O. Danay, T. McHugh, C. Bilbao, V. Rodov, E. Poverenov*	Efficient production of fungal chitosan utilizing an advanced freeze-thawing method; quality and activity studies	***Food Hydrocolloids***	:	Joint
Published	Reviewed	*C. Bilbao-Sainz, B.-S. Chiou, T. Williams, D. Wood, W.-X.Du, I. Sedej, Z. Ban, V. Rodov, E. Poverenov, T. McHugh*	Vitamin D-fortified chitosan films from mushroom waste	***Carbohydrate Polymers***	:	Joint
Submitted	Reviewed	*Elena Poverenov, Yana Zaicev, Viki Bar, Ofer Danay, Batia Horev, Cristina Bilbao-Sainz, Tara McHugh, Victor Rodov.*	Using chitosan from mushroom waste for preservation of agricultural food products; quality and storability enhancement of fresh-cut melons.	***Food Chemistry***	:	Joint
Submitted	Reviewed	*Cristina Bilbao-Sainz, Bor-Sen Chiou, Kaylin Punotai, Donald Olson, Tina Williams, Delilah Wood, Victor Rodov, Elena Poverenov, Tara McHugh.*	Layer-by-layer alginate and fungal chitosan based edible coatings applied to fruit bars	***Journal of Food Science***	:	Joint

Paper 1

Published in *Carbohydrate Polymers*, 2017, *167*, 97-104**.**

Vitamin D-fortified chitosan films from mushroom waste

Cristina Bilbao-Sainz[a], Bor-Sen Chiou[b], Tina Williams[b], Delilah Wood[b], Wen-Xian Du[a], Ivana Sedej[a], Zhaojun Ban[c], Victor Rodov[c], Elena Poverenov[c], Yakov Vinokur[c], Tara McHugh[a]

[a] Healthy Processed Foods Research, U.S. Department of Agriculture, Albany, CA, United States

[b] Bioproducts Research Unit, U.S. Department of Agriculture, Albany, CA, United States

[c] Postharvest and Food Science Institute, Agricultural Research Organization, The Volcani Center, Rishon LeZion 50250, Israel.

ABSTRACT

Brown mushroom (*Agaricus bisporus*) stalk bases from mushroom waste were treated with UV-B light to rapidly increase vitamin D_2 content. Chitin was also recovered from this waste and converted into chitosan by N-deacetylation. FTIR spectra showed that the mushroom chitosan were similar to chitosan from animal sources. Chitosan films were prepared using high molecular weight (HW), low molecular weight (LW) and fungal chitosan. UV-B treated mushroom particles were also incorporated into fungal chitosan films. The fungal chitosan films showed similar density, porosity and water vapor barrier properties to the LW and HW chitosan films. However, fungal chitosan films were more hydrophobic and less flexible than the LW and HW chitosan films. Addition of mushroom particles did not significantly affect mechanical or water barrier properties of the fungal chitosan films.

Keywords: Mushroom, chitosan, UV light, film, vitamin D

1. Introduction

The National Center for Health Statistics found that more than 70% of American adolescents and adults were deficient in Vitamin D in 2004 (Ginde, Liu, & Camargo, 2009). The major health problems resulting from vitamin D deficiency are rickets in children and osteomalacia in adults. Recently, there has been intense interest in the role of vitamin D in a variety of nonskeletal medical conditions. For instance, vitamin D has been linked to a significant reduced risk of breast cancer, colon cancer, prostate cancer, autoimmune disease

and cardiovascular disease (Holick, 2004). Vitamin D intake comes naturally from exposure to sunlight since humans are capable of converting cholesterol present in the epidermis to vitamin D_3 (Holick et al., 1980). However, sunlight exposure might be inadequate to produce the amount of vitamin D required to maintain good health. Consequently, other sources of vitamin D, such as foods, are required to supplement those derived from sunlight.

The Food and Nutrition Board of the Institute of Medicine recommends a dietary allowance (DRA) for vitamin D of 600−800 IU (15−20 μg) per day (Institute of Medicine, 2011). Vitamin D exists in two distinct forms: vitamin D_3 (cholecalciferol), present mainly in oily fish and bovine liver, and vitamin D_2 (ergocalciferol), present in yeasts and mushrooms (Shrapnel & Truswell, 2006). Commercial mushroom growers have recently incorporated UV light into their production processes to enhance the vitamin D content of mushrooms from almost nondetectable levels up to 6000−8000 IU/g (150−200 μg/g). Following UV light exposure, ergosterol (the most abundant phytosterol in mushrooms) undergoes photolysis to produce previtamin D_2, which then slowly isomerizes to vitamin D_2 via a thermal reaction (Phillips et al., 2011; Villares, Mateo-Vivaracho, Garcia-Lafuente, & Guillamon, 2014). Vitamin D_2 produced from UV irradiated mushrooms has been shown to be bioavailable, with an increase in blood levels of 25-hydroxyvitamin D, which is a biomarker for a person's vitamin D status. This increase is similar to those from someone ingesting fortified food, vitamin D_2 supplement and pharmaceutical formulation (Keegan, Lu, Bogusz, Williams, & Holick, 2013; Koyyalamudi, Jeong, Song, Cho, & Pang, 2009; Urbain, Singler, Ihorst, Biesalski, & Bertz, 2011). Also, mushrooms represent a potential source of vitamin D that is not derived from animals and can be considered vegan.

Mushrooms can also be considered as an alternative non-animal source for chitosan production. Chitin and its derivative, chitosan, are abundant and renewable biopolymers found in nature. Chitosan is a natural, biocompatible and biodegradable polycationic polysaccharide that possesses antimicrobial activity and filmogenic properties. Chitosan-based films have shown antimicrobial activity, for instance, chitosan nanocomposite films loaded with silver and zinc oxide nanoparticles as well as bionanocomposites based on chitosan/poly(vinylalcohol)/titanium nanoparticles and chitosan/calcium silicate nanocomposites doped with $AgNO_3$ have shown good antimicrobial activity against gram positive, gram negative and fungi (Youssef, Abou-Yousef, El-Sayed and Kame, 2015; Youssef, El-Sayed, Salama, El-Sayed and Dufresne, 2015; El-Nahrawy, Ali, Abou Hammad, and Youssef, 2016). Currently, most of the commercial chitosan is derived from crustaceans, such as shrimps and crabs. However, mushroom cellular walls have high chitin content,

which might be transformed into chitosan through a deacetylation reaction. Several mushrooms genera including *Agaricus, Pleurotus, Ganoderma* and *Lentinula* have been suggested as alternative chitosan sources (Kannan, Nesakumari, Rajarathinam, & Singh, 2010; Ospina et al., 2015; Yen & Mau, 2007)

Moreover, mushroom industry waste might be utilized to produce vitamin D and chitosan rather than using high-quality mushrooms (Wu, Zivanovic, Draughon, & Sams, 2004). During harvesting of mushrooms, their stalk bases are generated as a waste product. These bases comprise approximately 25% to 33% of the weight of fresh mushrooms and are normally used as low-economic value animal feed and compost (Chou, Sheih, & Fang, 2013).

Our hypothesis is that brown mushroom (*Agaricus bisporus*) waste can be used to obtain chitosan and develop nutritionally fortified films. In this study, we extracted chitosan from the stalk bases of brown mushrooms. We compared physicochemical properties of fungal chitosan with high molecular weight (HW) and low molecular weight (LW) chitosan from animal origin. We treated the mushroom stalk bases with UV-B light to increase vitamin D_2 content. We then cast and characterized HW and LW chitosan films as well as fungal and fungal chitosan films containing UV treated mushroom particles.

2. Experimental

2.1. UV treatment of mushrooms stalks

Brown mushroom (*Agaricus bisporus*) byproducts were obtained from Monterey Mushrooms, Inc. (Watsonville, CA) on the day of mushroom harvest. Mushrooms were medium sized, with cap diameters typically ranging from 3 to 4.5 cm as designated by the facility. Each batch of mushrooms was harvested in the morning by trained individuals. Mushroom stalks were collected by cutting off the mycelium from the stalk bases. Stalks were then stored at 5 °C overnight. One day after harvesting, the stalks were washed, cut into pieces and treated with UV-B radiation.A Uvitron UV Conveyor 40 dual-lamp curing system with two SunRay 400 SM UVB flood lamps (Uvitron International Inc., West Springfield, MA, USA) was used for the UV treatment. UV-B dose (energy) and peak intensity (power) were measured using an Uvicure Plus II radiometer (EIT Inc., Sterling, VA). The lamp height was set at 3.8 cm to achieve a peak intensity of 492 W/m^2. The conveyor speed was adjusted to achieve 1 J/cm^2 UV-B doses. During this experiment, the mushrooms were placed on the conveyor without regard to mushroom orientation and in a manner identical to that employed during a typical commercial production run.

After the UV-B treatments, mushroom stalks were placed flat in single layers on stainless steel trays and freeze dried in a Labconco Freezone 12 Freeze Dry System (Kansas city, MO, USA). After drying, the samples were ground to a fine powder with particle sizes smaller than 0.06 mm (Tyler screen size 28) and sealed under nitrogen in laminated PET bags (Impak corp., Los Angeles, CA, USA) for further analysis.

2.2. Vitamin D_2 analysis.

The vitamin D_2 analysis was performed in agreement with the official AOAC 2002.05 method (AOAC, 2007) comprising alkaline hydrolysis (saponification) of the material with subsequent extraction of the hydrolysate with 40% ethanol and n-heptane and HPLC analysis using vitamin D_3 as internal standard. The HPLC analysis was performed using a Prominence HPLC system (Shimadzu, Kyoto, Japan) with an Inertsil ODS-P C-18 RP column (GL Sciences, Japan), 250 ×4.9 mm I.D., and particle size 5 µm, flow rate 1 mL/min, injection volume 50 µL, column temperature 40°C, eluents: A (75% acetonitrile, 25% methanol) and B (100% ethanol). The gradient program was as follows: 0 to 11 min, linear gradient from 100% to 90% eluent A; 11 to 15 min, linear gradient from 90% to 20% eluent A; 15 to 19 min, linear gradient to 100% eluent B; 19 to 24 min, 100% eluent B; 24 to 32 min, linear gradient back to 100% eluent A. The detection was conducted using a SPD-M20A photodiode array detector at 264 nm. The vitamin D_2 peak was identified by comparison with authentic sample (Acros Organics, USA) and quantified using an internal standard of 10 µg vitamin D_3 (Acros Organics, USA).

2.3. Chitin extraction

Dried and ground mushroom stalk bases (100 g) were homogenized in 96 % ethanol. The supernatant was separated by centrifugation at 10,000 rpm for 15 min (Dupont Sorvall centrifuge RC-5C, Thermo Fisher Scientific, Waltham MA, USA), and the extraction-centrifugation cycle was repeated two more times. Then sodium metabisulfite solution (1000 ml of 0.5 % $Na_2S_2O_5$ dissolved in 5 % HCl) was added to the mushroom residue and left for 1 h at room temperature (23 ^oC). Afterwards, the residue was washed with deionized water by centrifugation until it reached a pH of 7. The mushroom residue was mixed with 1000 ml of 2 % NaOH and heated at 56 ^oC for 2 h. Then the mixture was centrifuged and the remains were washed 3 times with deionized water.

Sodium hydroxide solution (750 ml of 0.1 M NaOH) was added to the insoluble phase and then H_2O_2 was added gently to a final concentration of 3 %. The mixture was continually

stirred at 45 ^{o}C for 0.5 h. Crude chitin was obtained after centrifugation and washing until it reached a pH of 7

2.3.1. Chitin deacetylation

An aqueous solution of sodium hydroxide (400 ml of 50 % NaOH) was added to the extracted chitin and the mixture was boiled for 2 h at 104 ^{o}C. Deionized water was continuously added to the original volume during the boiling process. Afterwards, the mixture was cooled to room temperature and the solid phase was separated by centrifugation at 10000 rpm for 15 min. Then HCl solution (1M) was added until the solution reached a pH of 8.5. The precipitated chitosan was collected by centrifugation at 10,000 rpm for 15 min, washed with deionized water until it reached a pH of 5.7 and dried by lyophilization. As a comparison to mushroom chitosan, low molecular weight chitosan (product number 448869) and high molecular weight chitosan (product number 419419) were purchased from Sigma-Aldrich (St. Louis, MO).

2.4. Molecular weight

The viscosity average molecular weight (M_v) was determined by using a TA Instruments (New Castle, DE) AR2000 rheometer. The chitosan samples were dissolved in 0.25M acetic acid/0.25M sodium acetate solution. The LW and HW samples were mixed using a stir bar overnight at room temperature, whereas the fungal chitosan sample was mixed using a stir bar for 48 h at room temperature. The sample was placed in a concentric cylinder and then equilibrated at 25^{o}C for 10 min before the start of each experiment. The viscosity of the sample was then determined at 25 ^{o}C by varying the shear rate from 0.1 to 400 s^{-1}. M_v was determined from the Mark-Houwink-Sakurada equation (Kassai, Arul, & Charlet, 2000):

$$[\eta] = 1.57x10^{-4} M_v^{0.79} \quad (1)$$

where $[\eta]$ is the intrinsic viscosity (dL/g).

2.5. Degree of deacetylation (DD)

The degree of acetylation (DA) was determined by an improved first derivative UV method reported by Wu & Zivanovic (Wu & Zivanovic, 2008). The degree of deacetylation (DD) of chitosan samples was calculated as: DD (%) =100–DA.

2.6. Fourier Transform Infra-red (FTIR) spectroscopy

FTIR analysis of HW, LW and fungal chitosan was determined using a NICOLET™ iS™ 10 FT-IR spectrometer fitted with a Smart iTR diamond ATR detector. Chitosan samples were first dispersed in KBr to form pellets. The absorption spectra of the samples were recorded between 500 and 4000 cm^{-1} at a resolution of 4 cm^{-1} using a total of 64 scans.

2.7. Thermogravimetric analysis

A Perkin–Elmer (New Castle, DE) thermogravimetric analyzer (TGA) Pyris 1 was used to characterize the thermal stability of the HW, LW and fungal chitosan samples. Each 4–6 mg sample was heated from 30 to 600 °C at a rate of 10 °C/min. The sample chamber was purged with nitrogen gas at a flow rate of 40 cm^3/min. Three replicates were tested for each sample.

2.8. Preparation of films

Four different film forming solutions were prepared: LW chitosan, HW chitosan, fungal chitosan and fungal chitosan containing mushroom particles. Solutions were prepared by dispersing 1.5% (w/v) chitosan in 0.7% (v/v) lactic acid. Glycerol (25% (w/w) of chitosan) was then added to the solution and magnetically stirred for 1 h. Additionally, UV treated mushroom particles were added to the fungal chitosan solution to provide 100% RDA of vitamin D_2/g dry solids. The film solutions were degassed by applying vacuum to prevent micro-bubble formation in the films. Glass casting plates (30 × 30 cm) with Mylar (Dupont, Hopewell, VA) covers were used for film casting. The solutions were cast onto plates using casting bars and the samples were allowed to dry at room temperature.

2.9. Density and porosity

The bulk densities of the different films were calculated from measurements of sample weight and volume. Each film was weighed to the nearest 0.0001 g, and its dimensions were measured with a micrometer to the nearest 0.001 mm. The true density for each film was measured using a gas pycnometer (AccuPyc II 1340 Series pycnometer;

Micromeritics Instrument Co., Norcross, GA). Porosity (ε) was calculated according to the following correlation:

$$\varepsilon = 1 - \frac{\rho_b}{\rho_t} \quad (2)$$

where ρ_t is the true density and ρ_b is the bulk density.

2.10. Scanning electron microscopy (SEM)

Film cross sections were prepared by dipping a film into liquid nitrogen followed by fracturing with a pre-chilled razor. The freshly fractured pieces were retrieved from liquid nitrogen and placed into a desiccator to dry to room temperature. The pieces were then mounted with their fractured surfaces facing up on a stub using carbon adhesive tabs (Ted Pella Inc., Pleasanton, CA). They were coated with gold-palladium in a Denton Desk II sputter coating unit (Denton Vacuum U.S.A, Moorestown, NJ). All samples were then viewed and photographed using a Hitachi S-4700 field emission scanning electron microscope (Hitachi, Japan) at 2 kV.

2.11. Dynamic vapor sorption

A dynamic vapor sorption analyzer DVS-1 (Surface Measurement Systems, Allentown, PA) was used to measure the water sorption isotherms of the films. Each 4 mg sample was hydrated at a specific relative humidity until the sample reached equilibrium. The sample was exposed to a humidity range of 0–98%. All measurements were performed at 25 °C.

The Guggenheim–Anderson–de Boer (GAB) model was used to fit the isotherm data:

$$\frac{M}{M_0} = \frac{C \cdot K \cdot a_W}{(1 - K \cdot a_W)(1 - K \cdot a_W + C \cdot K \cdot a_W)} \quad (3)$$

In this equation, M is the equilibrium moisture (g of water/g of dry mass), M_0 is the monolayer water content, a_w is the water activity, C is the Guggenheim constant, which represents the sorption heat of the first layer, and K is the sorption heat of the multilayer. The GAB model parameters were determined by polynomial regression using Microsoft Excel 2007. Three replicates were tested for each sample.

2.12. Water vapor permeability (WVP)

WVP of the films was determined using the gravimetric modified cup method based on ASTM E96-80. The films were mounted in poly(methyl methacrylate) test cells that had a 19.6 cm^2 opening. Deionized water was placed in the bottom of the cell to expose the film to a high water activity. The film in the cell was oriented with the shiny side (the film side originally in contact with the Mylar cover) facing down (toward the inner, high relative humidity (RH) environment of the cell). Average stagnant air gap heights between the water and the film were determined. The test cells were placed in a cabinet maintained at 25°C and pre-equilibrated to 0% RH using calcium sulfate desiccant. The cabinet also contained fans that achieved air velocities of 500 ft/min (152 m/min) to ensure uniform RH throughout the cabinets. The cells were then weighed eight times at greater than 2 h intervals. For each experiment, the relative humidity at the film's underside and corrected WVP was calculated by the WVP correction method, accounting for the water vapor concentration gradient through the stagnant air layer in the cell (McHugh, Avena-Bustillos, & Krochta, 1993). Film thickness was measured with a micrometer to the nearest 0.001 mm. At least eight replicates were tested for each film.

2.13. Tensile properties

An Instron Universal Testing Machine (model 1122, Instron Corp., Canton, MA) was used to determine Young's modulus (*E*), tensile strength (*TS*) and percentage elongation at break (*EL*) of the films at 21 °C according to ASTM D882-97. The films were cut to a dog bone shape with a rectangular midsection (100 mm long x 15 mm wide) flaring to 25 mm x 35 mm sections on each end. Film thickness was measured with a micrometer to the nearest 0.001 mm. The films were conditioned at 50% RH for 72 h before each test. . A 100 N load cell was used and the extension rate was set at 25 mm/min. Ten replicates were tested for each film.

3. Results and discussion

3.1. UV-B treatment of mushrooms stalks.

Application of UV-B light to brown mushroom (*Agaricus Bisporus*) stalk bases at an average exposure of 1 J/cm^2 produced bases containing 91.5 ± 1.6 μg vitamin D_2/g on a dry weight (d.w.) basis. Similar vitamin D_2 values of 157μg/g were found for sliced brown mushrooms exposed to 2.8 J/cm^2 of UV-B irradiation for 10 min (Sapozhnikova, Byrdwell, Lobato, &

Romig, 2014). In comparison, whole brown mushrooms have vitamin D_2 concentrations between 0.0003-0.0008 μg/ g (Phillips et al., 2011). This indicated the effectiveness of UV-B irradiation for increasing vitamin D_2 content in mushrooms. Vitamin D_2 is found at a significantly higher concentration in the caps than in the stalks due to higher ergosterol concentration in the caps (Shao, Hernandez, Kramer, Rinker, & Tsao, 2010). However, the vitamin D_2 content found in one gram of treated mushroom stalks is still 4.5 to 6 times the RDA for vitamin D recently established by the Food and Nutrition Board of the Institute of Medicine (Institute of Medicine, 2011).

3.2. Chitosan molecular characteristics

The chitosan samples had significantly different viscosity average molecular weights (Mv): 250 kDa, 83 kDa and 21 kDa for HW-CH, LW-CH and fungal CH respectively, indicating different degrees of polymerization. Also, the different samples had different degrees of deacetylation: 75.5% ± 16.4%, 86.7% ± 10.6% and 91.4% ± 8.1% for HW-CH, LW-CH and fungal CH respectively. In a previous study, Yen and Mau (2007) extracted chitosan from shiitake stipes and found that it had an average MW of 382,730 Da and a DD of 90.2%. Other authors extracted chitosan from Aspergillus niger mycelium with an average Mw of 190,000 g/mol and 72.9 % DD (Muñoz, Valencia, Valderruten, Ruiz-Durántez & Zuluaga, 2015). The difference in molecular weight values might be due to different fungal sources and different N-deacetylation reaction times. Prolonged reaction times decrease Mv while increase the DD of chitosan molecules.

3.3. FTIR spectroscopy

The FTIR spectrum of fungal chitosan was similar to those of commercial LW and HW chitosan and other extracted fungal chitosans (Di Mario, Rapanà, Tomati, & Galli, 2008; Ospina et al., 2015) This is shown in Fig. 1. The different chitosan samples contained the same functional groups with variations in corresponding peak values.

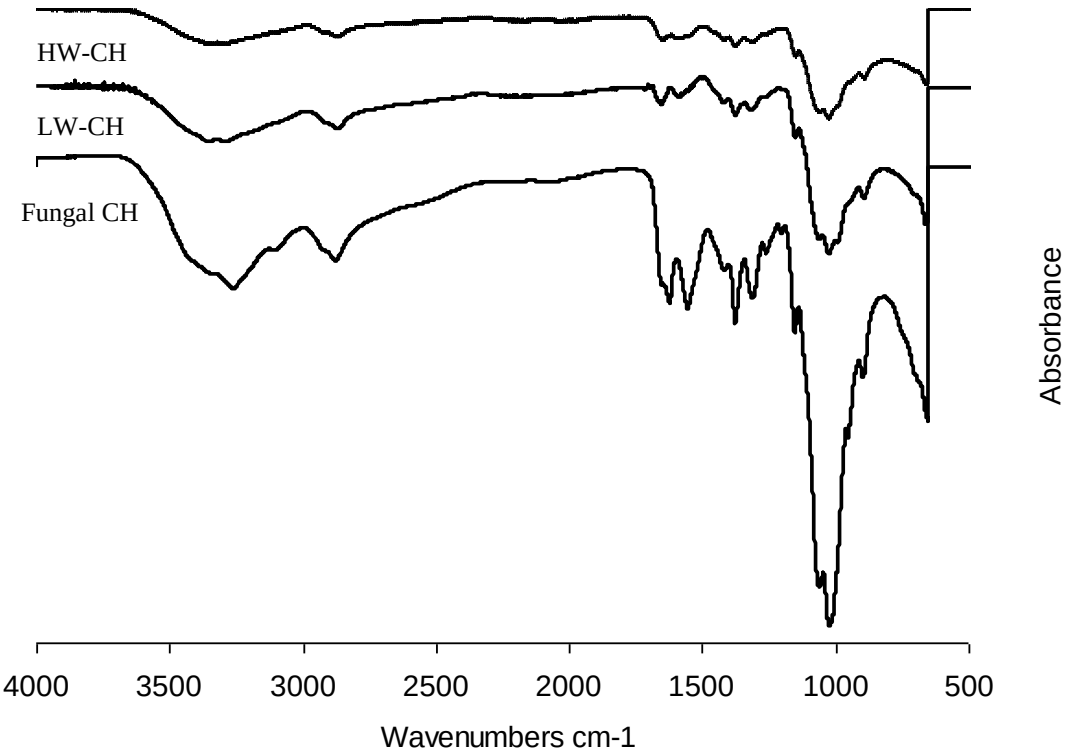

Fig. 1: FTIR spectra of animal-source HW chitosan, animal-source LW chitosan and fungal chitosan.

Table 1 compares the values of characteristic bands for each chitosan spectrum. The FTIR spectrum of fungal chitosan shows a peak at 1555 cm^{-1}, which is attributed to the bending vibrations of the amide II band (N–H). The absorption peak at 1312 cm^{-1} corresponds to C-N stretching, whereas the peak values of 1655 and 1623 cm-1 correspond to carbonyl stretching –C = O (amide I). Also, a broad peak between 3200 and 3570 cm^{-1} can be attributed to O–H and N–H stretching vibrations. The peaks at 2926 cm^{-1} and 2875 cm^{-1} correspond to stretching vibration of CH_3 and CH_2, respectively. The peak values of 1060 and 1023 cm^{-1} are attributed to C-O stretching. Additionally, the peak at 894 cm^{-1} correspond to the peaks of β-(1,4) glycosidic in chitosan (Di Mario, Rapanà, Tomati, & Galli, 2008; Ospina et al., 2015; Tajdini, Amini, Nafissi-Varcheh, & Faramarzi, 2010).

Table 1: Comparison of FTIR bands for fungal chitosan and animal-source HW and LW chitosans.

Molecule	HW-CH (cm^{-1})	LW-CH (cm^{-1})	Fungal CH (cm^{-1})

Group tension –OH	3360	3361	3364
Group tension –N-H	3288	3293	3261
Group tension C-H	2926 and 2878	2924 and 2871	2926 and 2875
Amide I	1653	1660	1655 and 1623
Doubling the group –NH2	1588	1588	1555
Amide III	1313	1315	1312
Tension C-O	1059 and 1025	1060 and 1024	1060 and 1023
Anomeric group CH tension	893	891	894

3.4. Thermal analysis

The thermogravimetric curve of fungal chitosan exhibited a three step mass loss, whereas the LW and HW chitosan samples showed a two step mass loss. The degradation temperature, mass loss and residues for the different chitosan samples are shown in table 2.

Table 2: Thermal parameters of different chitosan extracted from TGA thermographs

Chitosan type	Peak T (°C)			Mass loss (%)			Residue after 900 °C (%)
	1st peak	2nd peak	3rd peak	1st peak	2nd peak	3rd peak	
HW-CH	51.2^{b} (1.4)	288.2^{a} (0.7)		9.6^{a} (0.4)	40.0^{a} (0.7)		27.5^{a} (0.1)
LW-CH	71.3^{a} (3.3)	285.3^{a} (0.9)		10.7^{a} (0.4)	37.1^{a} (1.0)		21.5^{a} (15.0)
Fungal CH	46.8^{b} (1.7)	274.6^{b} (2.4)	325.8 (18.9)	9.1^{a} (0.4)	30.4^{b} (0.7)	19.1 (4.6)	16.2^{a} (14.1)

Different superscripts in the same column indicate 95% significant differences among chitosan polymers.

The HW chitosan sample showed an initial mass loss step between 35 and 100 °C. This corresponded to the loss of adsorbed and bound water (Yeh et al., 2006). The second mass loss step occurred between 267 °C and 318 °C with a maximum degradation temperature of 288 °C. This step resulted in 40% total mass loss. This mass loss can be attributed to the degradation of the chitosan polymer. LW chitosan sample showed comparable thermal decomposition behavior to the HW sample. Similar results for the LW and HW chitosan samples were found by Zakaria, Izzah, Jawaid, & Hassan (2012). In that

study, the maximum degradation temperature for the second mass loss step was found to be 281 °C and the samples had 33 % residue. In comparison, the fungal chitosan sample showed mass loss in three steps. The first mass loss step occurred between 35 and 100 °C due to loss of absorbed water. In this step, the mass loss reached 9.1%. The second step occurred between 240 °C and 295 °C with a maximum degradation temperature of 275 °C. During this step, the sample lost 30.4 % of its mass. These results indicated that the fungal chitosan sample was slightly less thermally stable than the LW and HW chitosan samples. The third mass loss step occurred between 295 and 356 °C with 19.1 % total mass loss. Ospina and collaborators (2015) characterized thermal degradation of *Ganoderma lucidum* mushrooms and also found mass loss occurring in three steps. They attributed the two last mass loss steps to saccharide degradation in the molecular structure of organic material and to a further final degradation of the organic material.

3.5. Physical and microscopic properties of chitosan-based films

No significant differences were observed in the bulk density, true density and porosity of LW, HW and neat fungal chitosan films (Table 3).

Table 3: Thickness, density and porosity of chitosan based films

Film type	Thickness (mm)	Bulk density ρ_b (g/cm^3)	True density ρ_t (g/cm^3)	Porosity ε (%)
HW-CH	0.028 ± 0.001	1.33 ± 0.04 ^{a}	1.42 ± 0.01 ^{a}	6.10 ± 3.01 ^{b}
LW-CH	0.025 ± 0.004	1.25 ± 0.09 ^{a}	1.42 ± 0.01 ^{a}	12.09 ± 6.18 ^{b}
Fungal CH	0.021 ± 0.002	1.34 ± 0.03 ^{a}	1.43 ± 0.01 ^{a}	8.42 ± 4.77 ^{b}
Fungal CH/Mush	0.029 ± 0.004	1.00 ± 0.11 ^{b}	1.42 ± 0.00 ^{a}	29.60 ± 6.98 ^{a}

Different superscripts in the same column indicate 95% significant differences among films.

Similar bulk density values were reported for chitosan films containing 25% and 30% glycerol (Park and Zhao 2004; Siripatrawan & Harte 2010). In comparison, the film containing mushroom particles had lower bulk density than the other films, indicating the presence of voids. These voids resulted in a porosity value of 30 % for the film containing

particles. In comparison, porosity values for LW, HW and neat fungal chitosan films varied between 6 and 12%.

SEM micrographs of the cross-sections of LW and HW chitosan films showed a non-porous, layered pattern with uniform distribution and cracks mainly on the air side surface (Fig. 2A, 2B, 2C and 2D). Layered structure of pure chitosan films had been attributed to the orientation of chitosan chains in the matrix structure and to the evaporation front during drying (Kurek et al., 2012). The fungal chitosan film appeared more heterogeneous, but also contained cracks (Fig. 2E and 2F). In comparison, the fungal chitosan film containing mushroom particles showed some discontinuities with the presence of small holes (Fig. 2G and 2H). The presence of voids also made this film thicker than the others, with this film being 36% thicker than the neat fungal chitosan film (Table 3).

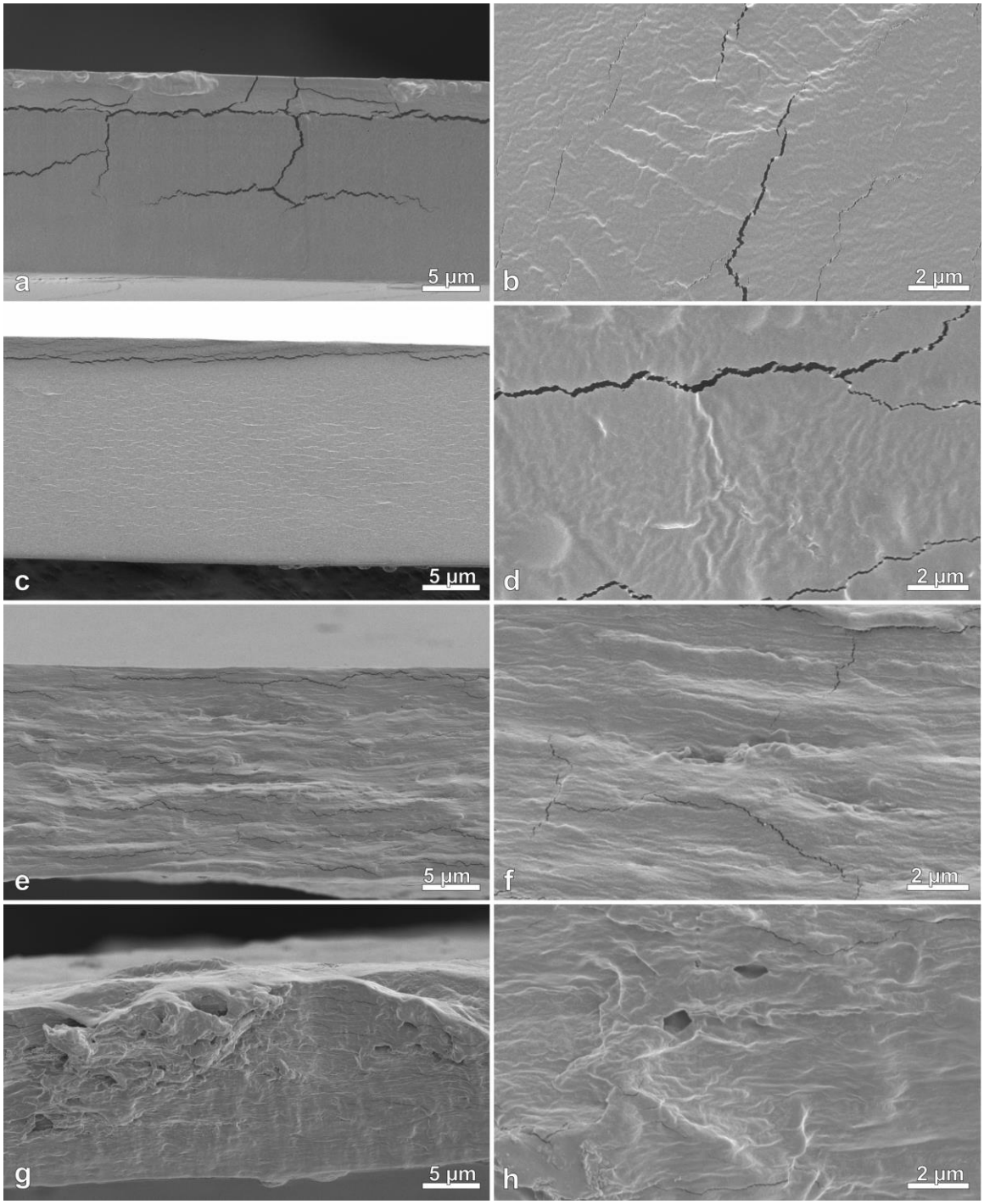

Fig 2: SEM micrographs of HW-CH film (a and b), LW-CH film (c and d), fungal CH film (e and f) and fungal chitosan film containing mushroom particles (g and h).

3.6. Water affinity of chitosan based films

The adsorption isotherms of the chitosan-based films and the mushroom particles at 25 °C are displayed in Fig. 3.

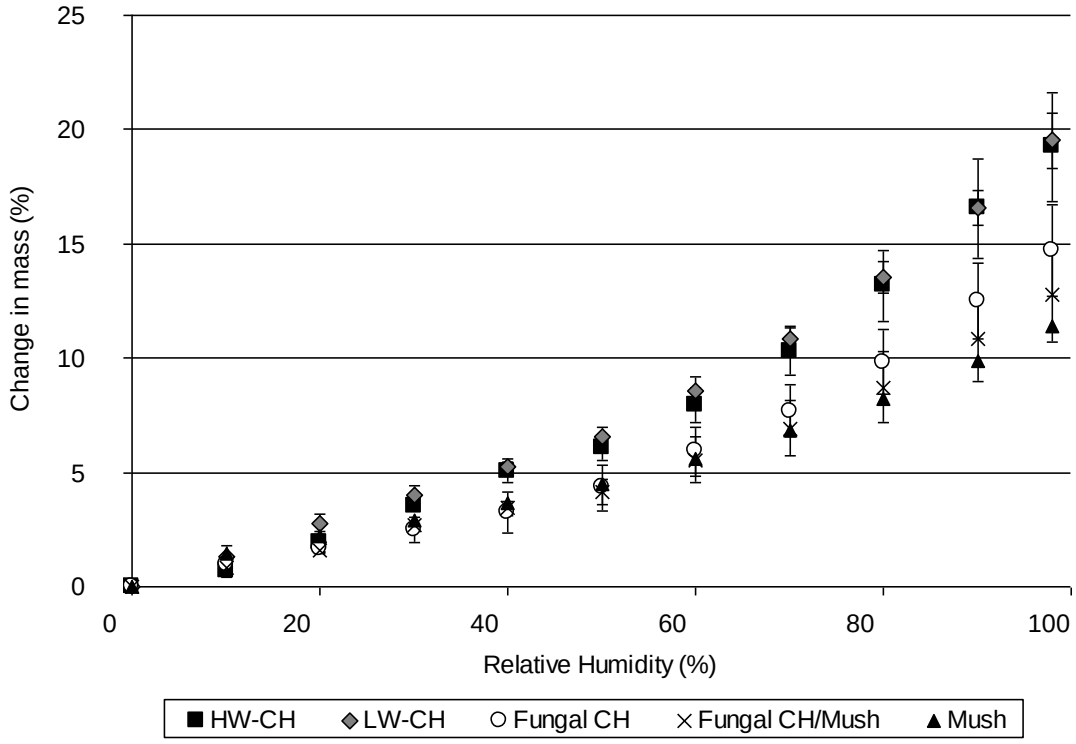

Fig. 3: Sorption isotherms for chitosan based films and mushroom particles.

The films and particles followed a type III isotherm (Brunauer, Deming, & Teller, 1940). These isotherms showed a slow initial increase in moisture content with relative humidity (RH) up to 50%. After this, the samples adsorbed water at a faster rate with eventual swelling and solubilization. The LW and HW chitosan films had comparable water sorption values, indicating molecular weight had little effect on chitosan sorption (Fig. 3). In comparison, the neat fungal chitosan film had lower water sorption values throughout the entire relative humidity range than those of LW and HW chitosan films (Fig. 3). This indicated that the neat fungal chitosan film was more hydrophobic than the other films and had lower equilibrium moisture contents. The incorporation of mushroom particles led to a further decrease in water sorption values due to the lower hygroscopic nature of the mushroom particles (Fig. 3).

The GAB model fit the isotherm data well in the 0–98% RH range (R^2>0.94). Fungal chitosan was less hygroscopic than the LW and HW chitosan samples, resulting in lower monolayer water content (M_0). M_0 indicates the amount of water that is strongly adsorbed to specific sites. The M_0 value for the fungal chitosan film was 4.1 ± 1.8 g/100g compared to 12.3 ± 2.4 and 11.3 ± 1.7 g/100g for HW and LW chitosan films, respectively. The M_0 value for the fungal chitosan film containing mushroom particles was 4.2 ± 1.0 g/100g.

3.7. Water vapor permeability of chitosan-based films

All chitosan films had comparable water vapor permeability (WVP) values with no significant differences ($p>0.05$). This is shown in Table 4, which includes WVP, water vapor transmission rate (WVTR), film thickness and relative humidity values at the underside of the films.

Table 4: Water barrier properties of chitosan-based films

Film type	Thickness (mm)	RH (%) film underside	WVTR ($g/h.m^2$)	WVP ($g.mm/kPa.h.m^2$)
HW-CH	0.036 ± 0.005	76.8 ± 5.3	52.0 ± 2.1 [b]	0.74 ± 0.10 [a]
LW-CH	0.037 ± 0.004	77.5 ± 4.5	49.8 ± 0.5 [b]	0.70 ± 0.07 [a]
Fungal CH	0.025 ± 0.002	74.2 ± 3.9	60.5 ± 4.0 [a]	0.63 ± 0.08 [a]
Fungal CH/Mush	0.031 ± 0.002	74.9 ± 5.8	56.2 ± 1.8 [a]	0.71 ± 0.02 [a]

WVTR: water vapor transmission rate. WVP: water vapor permeability. CH: chitosan. Mush: Mushroom particles. Different superscripts in the same column indicate 95% significant differences among films.

The WVP of LW and HW chitosan films were 0.697 and 0.745 $g.mm/kPa.h.m^2$, respectively. These values were within the range of those reported for other chitosan films (Hwang, Kim, Jung, Cho, & Park, 2003; Park, Marsh, & Rhim, 2002). Previous studies also indicated that molecular weight and degree of deacetylation did not significantly affect the WVP of chitosan films (Chen & Zhao, 2012; Hwang, Kim, Jung, Cho, & Park, 2003). Also, the LW and HW chitosan films had similar sorption isotherm (Fig. 3), film density (table 3) and film porosity (table 3), indicating similar water affinity as well as similar open space within the film structure for water molecules to pass through. In comparison, the fungal chitosan film had a slightly lower WVP value (0.631 $g.mm/kPa.h.m^2$) compared with LW and HW chitosan films due its lower water affinity (Fig. 3). However the differences in WVP values between these films were not statistically significant. Also, the fungal chitosan film

containing mushroom particles had a higher WVP value than the neat fungal chitosan film. This might be due to the film containing particles having pores, resulting in poorer water vapor barrier properties (table 3).

3.8. Tensile properties of chitosan-based films

The chitosan films exhibited the stress-strain behavior of ductile polymers. The tensile strength (TS), Young's modulus (E) and percent elongation at break (*E*L) are summarized in Table 5.

Table 5: Mechanical properties of chitosan-based films

Film type	TS (MPa)	E (kPa)	EL (%)
HW-CH	5.17 ± 0.86 [a]	163.0 ± 26.0 [a]	65.7 ± 5.2 [a]
LW-CH	2.12 ± 0.86 [c]	44.9 ± 15.0 [b]	56.9 ± 9.5 [a]
Fungal CH	3.76 ± 0.94 [b]	114.8 ± 50.6 [a]	36.5 ± 3.5 [b]
Fungal CH/Mush	4.31 ± 1.77 [ab]	118.6 ± 66.8 [a]	30.2 ± 4.6 [b]

TS: Tensile stress. E: elastic modulus. EL: elongation at break. CH: chitosan. Mush: Mushroom particles. Different superscripts in the same column indicate 95% significant differences among films.

The HW chitosan film had the highest tensile strength value of 5.2 MPa, whereas the fungal and LW chitosan films had values of 3.8 and 2.1 MPa, respectively. Young's modulus of the films followed the same trend as that of tensile strength. The addition of mushroom particles did not significantly affect the film's modulus and strength values. Previous studies had shown that tensile strength of films depended on molecular weight of chitosan chains with higher molecular weight chains having higher tensile strength values (Chen and Zhao, 2012; Leceta, Guerrero, & de la Caba, 2013). This was due to the higher molecular weight chains forming more hydrogen bonds between hydroxyl and amino groups during the film formation process (Uragami, Matsuda, Okuno, & Miyata, 1994). Also, the HW chitosan film was the most flexible (EL = 66%), followed by the LW chitosan film (EL = 57%) and the fungal

chitosan film (EL = 36 %). Rhim, Weller, & Ham (1998) reported a comparable EL value of 58.3 % for chitosan films prepared with lactic acid. Previous studies found that elongation at break also increased in value for an increase in chitosan molecular weight (Chen and Hwa, 1996; Chen and Zhao, 2012). The authors attributed this result to an increase in chain entanglements during film formation. The effects of molecular weight on elongation at break values in this study agreed with the previous results. Also, addition of mushroom particles resulted in a slight decrease in the elongation at break value to 30%. This might be due to the mushroom particles interrupting formation of crystalline structure and weakening intermolecular hydrogen bonding of the chitosan chains in the matrix.

4. Conclusions

This study demonstrated that low value mushroom waste could be used to develop chitosan films fortified with vitamin D_2. Mushroom stalk bases treated with UV-B light contained 4.5 to 6 times the RDA/g of vitamin D_2. The freeze-dried and ground mushroom powder retained its vitamin D_2 and could be further used as an ingredient to prepare nutritionally fortified chitosan films. Also, mushroom stalk bases could be an alternative source for isolating chitosan with similar properties to animal-based chitosan. In addition, fungal chitosan is a good film-forming material that possesses film properties similar to those of chitosan films from animal origin.

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Paper 2

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Efficient production of fungal chitosan utilizing an advanced freeze-thawing method; quality and activity studies

Zhaojun Ban[a,b], Batia Horev[a], Roi Rutenberg[c,a], Ofer Danay[d], Cristina Bilbao[e], Tara McHugh[e], Victor Rodov[a], Elena Poverenov[a,*]

[a] Postharvest and Food Science Institute, Agricultural Research Organization, The Volcani Center, Rishon LeZion 50250, Israel.

[b] Key Laboratory of Agricultural Products Chemical and Biological Processing Technology of Zhejiang Province, School of Biological and Chemical Engineering, Zhejiang University of Science and Technology, Hangzhou, Zhejiang 310023, People's Republic of China (present address).

[c] Institute of Biochemistry, Food Science and Nutrition, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel

[d] MIGAL Galilee Research Institute LTD, Kiryat Shemona 11016, Israel.

[e] Western Regional Research Center, United States Department of Agriculture, Agricultural Research Service, Albany, CA, 94710, USA

* Corresponding author: Dr. Elena Poverenov; e-mail: elenap@volcani.agri.gov.il;
Tel: 972-3-9683354; Fax: 972-3-9683692.

Abstract

Fungal chitosan was produced from brown *Agaricus bisporus*, white *Agaricus bisporus*, and *Pleurotus ostreatus* mushrooms (commercially known as brown portobello, white protobello, and oyster mushrooms, respectively) by utilizing a classic alkali deacetylation protocol (PI), as well as a new protocol (PII) that involves a freeze-thawing cycle. The new protocol, PII led to an up to twofold increase in the obtained chitosan's yield, a more efficient deacetylation process that resulted in up to threefold decrease of acetylated units and whiter color. For instance using PII protocol, 1 g. of dry *Pleurotus ostreatus* mushrooms yielded 44.3 mg of chitosan that contains only 14.8 % of acetylated units and has a whiteness index of 80.3 For comparison, using classic PI protocol, 1 g. of dry *Pleurotus ostreatus* mushrooms yielded 22.4 mg of chitosan that contains 44.1 % of acetylated units and has a whiteness index of 75.7. Gel permeation chromatography, FTIR, NMR, UV, elemental analysis, X-ray diffractometry, and thermogravimetric analysis were all used to characterize the prepared fungal chitosan samples and examine their physical properties (viscosity, color, hydrophobicity, and solubility). Microbiological studies have revealed the yielded chitosan samples have demonstrated biological activity against Gram-positive *Bacillus subtilis*, Gram-negative *Escherichia coli* bacteria, and *Saccharomyces cerevisiae* yeast. In this paper, the prepared fungal chitosans were also compared with the crustacean-sourced commercial chitosan along all the studies. It can be concluded that PII protocol allows formation of fungal chitosan which properties do not concede to the properties of the crustacean chitosan. This study therefore presents an efficient method for the production of non-animal sourced chitosan, which is extremely desired in the food industry because it overcomes the limitations of animal chitosan and allows to expand usage of this material. In addition, the presented freeze-thaw cycle technique can also be used to yield and modify other edible hydrocolloids.

Keywords: Non-animal chitosan; freeze-thawing method; fungal; antimicrobial; edible hydrocolloids.

Introduction

Chitosan is a cationic hydrocolloid produced by the deacetylation of chitin, an abundant natural polysaccharide. It has attracted significant interest due to its versatile biological activities, endowment with antimicrobial properties, ability to elicit natural innate defense responses, and binding abilities to heavy minerals and fats (Elsabee & Abdou, 2013; Xia, Liu, Zhang & Chen, 2011). Due to its intrinsic bioactivity, biodegradability, biocompatibility, film-forming capacity, and lack of human toxicity, chitosan is widely applied in food products as a beneficial additive and an active edible coating (Elsabee & Abdou, 2013). It has been used in medicine for drug delivery and tissue engineering (Ding et al., 2012; Agnihotri, Mallikarjuna & Aminabhavi, 2004), and in agriculture as a natural seed treatment, plant growth enhancer, and an ecologically friendly biopesticide (Linden et al., 2000).

Currently most of commercial chitosan is produced from crustaceans (such as crabs and shrimp) by deacetylating chitin, a polymer present in their exoskeleton (Ghormade, Pathan & Deshpande, 2017). As an edible product that originates from an animal source, chitosan and chitosan-containing food products are unappealing to vegetarians, vegans and people who do not consume crustacean-sourced products due to medicinal, religious or other restrictions (Dunham, 2012; Regenstein, Chaudry & Regenstein, 2003). Mushroom cell walls can be considered as an alternative non-animal source for the production of chitosan. World mushroom production carries millions of tons of unavoidable associated waste that can be harnessed for the production of fungal chitosan (Wu, Zivanovic, Draughon & Sams, 2004), making it a high current research interest (Abdel-Gawad, Hifney, Fawzy & Gomaa, 2017; Alsaggaf, Moussa & Tayel, 2017; Kannan, Nesakumari, Rajarathinam & Singh, 2010; Muzzarelli et al., 2012).

The deacetylation process involves the removal *N*-acetyl groups from biopolymer chitin to produce chitosan. The deacetylation process is crucial for chitosan high quality, since degree of acetylation (% DA) determines its subsequent solubility, antimicrobial activity and other accompanied applicative features (Elsabee & Abdou, 2013). It is rather difficult to reach effective deacetylation by classic protocol, especially in the case of mushroom-sourced raw materials (Yen, Yang & Mau, 2009). In order to increase the application of fungal chitosan, new effective and feasible approaches that allow to enhance an efficacy of deacetylation process without increasing an amount of alkali agent and without using higher temperatures or other severe conditions are needed.

The freeze-thaw treatment of a polymer includes alternate freezing and thawing procedure. Ice crystals affect intramolecular and intermolecular hydrogen bonds of the polymer

disorganize its structure and making it more available for reactions (Liu et al., 2010). Lamarque et al. (2005) have designed a freeze-pump out-thaw (FPT) cycle in the presence of 50 % NaOH, and have succeeded in the deacetylation of α- and β-chitins. However, the high concentration of the alkali reagent, as well as a repeated degassing step ("pumping out") needed for each cycle, have ultimately inhibited this interesting protocol's applicability.

In the present research, we have aimed to increase the yield and quality of fungal chitosan by applying an applicable, cost-effective and safe method. Chitosan that satisfies the following parameters: degree of deacethylation at least 75 %, white color and antimicrobial activity is usually considered as a qualitative one. A classic alkali deacetylation method (PI) and a freeze-thawing method (PII) were utilized for the production of fungal chitosan from brown *Agaricus bisporus*, white *Agaricus bisporus*, and *Pleurotus ostreatus* (commercially known as brown portobello, white protobello, and oyster mushrooms, respectively). The obtained chitosan was comprehensively analyzed and compared to commercial animal-sourced samples in terms of structural, spectroscopic, physical, and antimicrobial features.

2. Material and methods

2.1. Mushrooms

Three mushroom types including brown *A. bisporus*, white *A. bisporu*s, and *P. ostreatus* were provided by Dr. Ofer Danay (MIGAL-Galilee Research Institute, Kiryat Shmona, Israel). The fresh mushrooms were cut into small pieces and kept at -40 ^{o}C until use. The three mushroom types were also tested for their dry matter content that was higher in *P. ostreatus* (9.96±1.16 %) and brown *A. bisporus* (9.20±1.86 %) than in white *A. bisporus* muhrooms (7.76±0.30 %).

2.2. Preparation of chitosan

Commercial chitosan was purchased from Molekula (Newcastle upon Tyne, UK, Product No. 42000446). Three types of fungal chitosan, which included brown *A. bisporus* chitosan, white *A. bisporus* chitosan, and *P. ostreatus* chitosan, were extracted in our lab. The frozen mushroom materials were weighed and lyophilized, and the dry mushroom content was determined.

2.2.1. Chitin Extraction

Dried mushroom material (100 g) was homogenized in 96 % ethanol. The supernatant was separated by centrifugation at 10,000 rpm for 15 min (Sorvall RC-5C Plus, Thermo Fisher

Scientific, Waltham MA, USA), and the extraction-centrifugation cycles were repeated two more times. The pooled ethanolic extract could then be used for further ergosterol analyses (out of the scope of the present report). A sodium metabisulfite solution (1000 mL of 0.5 % $Na_2S_2O_5$ dissolved in 5 % HCl) was then added to the mushroom residue and left for 1 h at room temperature, and then washed with deionized water by centrifugation until the pH was neutral. The mushroom residue was then mixed with 1000 mL of 2 % NaOH and heated to 56 ^oC for 2 h. The mixture was then centrifuged and the remains were washed 3 more times with deionized water.

A sodium hydroxide solution (750 mL of 0.1 M NaOH) was added to the insoluble phase and H_2O_2 was then gently added to a final concentration of 3 % (v/v). The mixture was kept at 45 ^oC for 0.5 h with slow stirring. Centrifugation and washing cycles were performed until the pH was neutral and the crude chitin was then obtained and ready to deacetylate.

2.2.2. Chitin Deacetylation: Protocol I

An aqueous solution of sodium hydroxide (400 mL of 50 % NaOH) was added to the chitin as obtained from the above description, and the mixture was heated for 2 h at 104 ^oC, according to the temperature measurements at the reaction vessel. Deionized water was continuously added to maintain the original volume during the boiling process. Afterwards, the mixture was cooled to room temperature and the solid phase was separated by centrifugation at 10,000 rpm for 15 min. An HCl solution (1 M) was then added until the pH was 8.5. The precipitated chitosan was collected by centrifugation at 10,000 rpm for 15 min, washed with deionized water until the pH was neutral, and lyophilized. The yield for chitosan was expressed as mg of chitosan per 1 g of dry mushroom as an average of three replications.

2.2.3. Chitin Deacetylation Using Freeze-Thaw Cycles: Protocol II

Chitin was prepared as described above. An aqueous solution of sodium hydroxide (400 mL of 50 % NaOH) was added to it, and the mixture was frozen using liquid nitrogen, thawed at room temperature, and frozen again. After 4 freeze-thaw cycles, the solution was heated and kept at 104 ^oC for 10 min. The suspension was cooled to room temperature and a solid phase was separated by centrifugation at 10,000 rpm for 15 min. The following procedures for neutralizing, washing and separating the chitosan product were all identical to the described method in the Section 2.2.2.

2.3. Measurements of physicochemical characteristics

2.3.1. Degree of acetylation (% DA)

% DA was determined using the first derivative UV method (Wu & Zivanovic, 2008).

Standard preparation and formation of standard curve:

N-Acetyl-*D*-glucosamine (GlcNAc) and Glucosamine (GlcN) were prepared in 0.85 % (v/v) phosphoric acid at concentrations of 0, 10, 20, 30, 40, 50 ug/mL. Calibration curves were made by plotting the first derivative UV values at 203 nm as a function of GlcNAc and GlcN concentrations.

Sample preparation for DA determination:

Aliquots of 100 ± 10 mg sample were then heated in 20 mL of 85 % (v/v) phosphoric acid at 60 ºC with constant stirring until completely dissolved. 1 mL of clear solution was then taken and diluted to 100 mL with deionized water. The diluted solutions were incubated at 60 ºC for 2 h prior the UV measurement. The chitosan samples' % DA was calculated as

$$DA(\%) = \frac{\frac{m1}{203.21} \times 100}{\frac{m1}{203.21} + \frac{m2}{161.17}}$$

where: $m1$ is the mass of acetyl-glucosamine in 1 mL chitosan solution, calculated from the calibration curve by the corresponding H_{203}; $m2$ is the mass of glucosamine in 1 mL chitosan solution, calculated as $m2 = M - m1$. The mass of chitosan (M) in the 1 mL solution was simplified to calculate as $M = M1/20$, where $M1$ is mass of solid chitosan sample taken for analysis (100 ± 10 mg).

The formula can be transferred to

$$DA(\%) = \frac{167.17 \times m1 \times 100}{167.17 \times m1 + 10.16 \times (M1 - 20 \times m1)}$$

2.3.2. Intrinsic Viscosity

Chitosan samples were dissolved in a 0.2 M acetic acid / 0.1 M sodium acetate aqueous solution. Intrinsic viscosity [η] was measured with an Ubbelohde viscometer at 30 ± 0.1 °C and calculated by extrapolating a graph of reduced viscosity and inherent viscosity to zero concentration. The average of the two obtained intercept values was then calculated.

2.3.3 Gel Permeation Chromatography

Molecular weights and polydispersity indices of the modified chitosan products were determined using Gel Permeation Chromatography (GPC) consisting of a Waters Alliance system e2695 separation module (Waters Israel), equipped with a refractive index detector, model blue 2414. The mobile phase used was a 0.02 M acetic acid / 0.1 M sodium

acetate buffer with an addition of 10 mL of acetic acid to the 2.5 L buffer batch (pH 4.2), under isocratic elution for 30 min at a flow rate of 0.7 mL min^{-1}. The injection volume was 20 µL, and the temperature of both the detector and columns was 30 °C. Analyses were carried out using an ultra hydrogel column, 2000, 12 µm, 7.8 mm X 300 mm, 500 - 10M (Waters). Molecular weights were determined relative to a dextran standards kit (PSS Polymer standards service GmbH, Germany) with a molecular weight range of 4,400-401,000 Da. All data provided by the GPC system were collected and analyzed with the Empower 3 personal dissolution software.

2.3.4. Color measurement

Chitosan's powder color was measured using a Chroma Meter CR400 (Konica Minolta, Japan), and *L*, *a*, and *b* values were recorded. Each sample was individually measured in triplicate. Whiteness index (WI) was calculated based on the following equation reported by Hsu, Chen, Weng & Tseng (2003).

$$WI=100-\sqrt{(100-L)^2+a^2+b^2}$$

2.3.5. Solubility

Chitosan's solubility was estimated by a previously published method by Chung et al. with a minor modification (Chung, Kuo & Chen, 2005). Chitosan samples (0.1 g) were suspended in 5 mL of distilled water (with a measured pH of 6.5) in a 15 mL conical bottom culture tube and stirred at room temperature for 5 h. Undissolved portions were separated by centrifugation and washed with acetone, then dried under vacuum until their weight was constant. The soluble portion's weight was determined by the difference between the initial sample weight and the weight of the insoluble portion. The solubility of the samples was calculated as the average of three independent replications and expressed as g L^{-1}.

2.3.6. Fourier transform infrared (FTIR) spectroscopy

FTIR analyses were performed with a Bruker Tensor 27 FTIR spectrometer (Bruker, Billerica MS, USA). Samples were prepared by forming a pellet of mixed chitosan powder with anhydrous potassium bromide by a mass ratio of 1:100. Analyses were conducted between 400 and 4000 cm^{-1} with 100 scans averaged at a resolution of 4 cm^{-1}.

2.3.7. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded at 400 MHz using a Bruker AMX-400 NMR spectrometer. All spectra were recorded at 23 °C. ^{1}H NMR chemical shifts are reported in parts per million (ppm) and referenced to the residual hydrogen signal of the deuterated solvent (D_2O 4.79 ppm). Screw-cap 5 mm NMR tubes were used in the NMR follow-up experiments.

2.3.8 Thermogravimetric analysis (TGA)

Thermal properties were measured with a thermogravimetric analyzer (TGA Q500, TA Instruments). Samples were heated to 600 ^{o}C at a heating rate of 10 ^{o}C/min under nitrogen flow.

2.3.9 Water contact angle

Static contact angles for chitosan film samples were measured at ambient conditions using a Kruss Drop Shape Analyzer instrument, model DSA100S. Angles were measured within 1 s of contact, after placing 2 µL individual drops of deionized water on the film surface. For each sample, contact angles of three different positions on the surface were measured in triplicate and calculated by the sessile drop method, using the Advance software to consider the average value. Films were prepared by dissolving a chitosan powder in water (1.5 %w/v) with acetic acid (0.6 %v/v). Solutions were stirred for 1 h to achieve homogeneity and then poured (5 mL) into Teflon petri dishes with a 5 cm diameter, where they were left to spontaneously dry overnight in a chemical hood at room temperature.

2.3.10 Determination of nitrogen content

Content of nitrogen was determined using the Micro-Kjeldahl method. Briefly, 1 g chitosan samples were heated to 60 ^{o}C and grinded to 1 mm pellets. Pellets were then moved to beakers, where 10 mL of sulphuric acid 98 % were added, along with 35 mL of a 10 % hydrogen peroxide solution and 0.15 gr of Kjeldahl catalyst (a selenium reagent mixture). Beakers were kept at 400 ^{o}C for 90 min in a Buchi K-435 reflectometer. Samples were then cooled to room temp. over a course of 30 min and distilled for 4 min at a Buchi B-324 distiller with 60 mL of a 32 % sodium hydroxide solution and 50 mL DDW. Following the distillation, samples were titrated with a 0.1 N hydrochloric acid solution and 65 mL of a 2 % boric acid solution, using a Metrohm 719S titrator. Results are presented as % of the dry material (% DM).

2.3.11 X-ray diffractometry

X-Ray patterns for CS and the fungal–sourced samples were analyzed as film samples using a Bruker D8 advanced X-ray diffractometer. Samples were scanned between 2ϑ=10-90° with a scanning speed of 2°min^{-1}.

2.4. Evaluation of antimicrobial activity

2.4.1. Preparation of microorganism inocula

Bacillus subtilis ATCC-3610 and *Escherichia coli ATCC*-25922 were used as representative strains of Gram-positive and Gram-negative bacteria, respectively. They were subcultured into Difco LB Broth, Lennox (Becton Dickinson, Sparks MD) and grown overnight at 37 °C. The cultures were then centrifuged at 10,000 rpm for 2 min and the precipitate was resuspended in double strength LB broth (LB×2) and adjusted to OD 0.2 at 600 nm (Ultrospec 2100 pro, Amersham Biosciences, Piscataway NJ) to ca. 2×10^9 cells mL^{-1}. The bacterial suspensions obtained were further diluted with LB×2 to the density of approximately 2×10^4 cells mL^{-1}, serving as inoculation in antimicrobial activity tests. The inoculum densities were confirmed by plating their decimal dilutions onto LB agar medium. *Saccharomyces cerevisiae* YSC-2 Yeast, Baker yeast Type II was sub-cultured into NYDB (Nutrient Yeast Dextrose Broth) prepared as described by Dogra et al. (2015) and grown at 30 °C for 24 h. The inoculum of 2×10^4 yeast cells mL^{-1} was prepared as described above for bacterial cultures except for using double-strength NYDB medium (NYDB×2) instead of LB×2.

2.4.2. Antimicrobial activity determination

The antimicrobial effect of chitosan samples was determined via minimum inhibitory concentrations (MIC) using the broth micro-dilution method. A chitosan solution of 20 mg mL^{-1} in 0.5 % acetic acid was used as stock. The stock was diluted with the 2-fold dilution method using 0.5 % acetic acid, and further mixed at 1:1 with bacterial or yeast inocula to obtain a series of chitosan concentrations of 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16 and 0.08 mg mL^{-1} in LB or NYDB liquid media. All dilutions contained approximately 10^4 cells mL^{-1} and had a pH of 5.5 to 5.7. The dilutions were pipetted in triplicate into wells of 96-well plates (Cellstar®, Greiner Bio-One, Kremsmunster, Austria), 300 µL per well. The wells with a chitosan-free inoculated medium served as a positive control and the wells with a non-inoculated chitosan-free medium as a negative control. The growth kinetics of bacteria and yeast was monitored for 24 h at either 37 °C or 30 °C, respectively, as a change of optical

density at 600 nm using an EnSpire microplate reader (PerkinElmer, Waltham MA, USA). MIC values were defined as the lowest concentration of chitosan that completely inhibited any microbial growth. After a 24 h incubation, 100 µL aliquots from the wells showing no apparent growth were plated onto solid nutrient media in order to check bacteria or yeast recovery. The bacteria were incubated on LB agar at 37 ^{o}C for 24 h, while the yeast incubation was performed at 30 ^{o}C for 48 h on Difco potato dextrose agar (Becton Dickinson, Sparks MD) supplemented with 100 ppm of chloramphenicol (PDA+A). The lowest chitosan concentration showing no microbial recovery after the transfer onto a chitosan-free medium was defined as the minimum biocidal concentration (MBC).

2.5. Statistical analysis

All experiments were conducted in triplicates at the least. Microsoft Office Excel spreadsheets were used to calculate the means, standard deviations and 95 % *t* confidence intervals. Statistical analyses were carried out using the JMP software, version 10.0 (SAS Institute Inc., Cary, NC, USA), including a one-way analysis of variance (ANOVA) followed by the Tukey HSD pairwise comparison test.

3. Results and discussion

3.1. Effect of the preparation protocol on chitosan yield and quality

Chitosan was produced from brown *Agaricus bisporus* (brown *A. bisporus*), white *Agaricus bisporus* (white *A. bisporus*), and *Pleurotus ostreatus* (*P. ostreatus*) mushrooms using two different protocols; the classic alkali deacetylation protocol (PI), and a new protocol that involves freeze-thawing cycles (PII). Products from the two protocols were compared for their quality parameters, yields, degree of acetylation (% DA), and whiteness indices (Table 1). Notably, the degree of acetylation was significantly decreased by the freeze-thawing method (PII) in all of the inspected mushroom types, leading to a % DA of 28 - 15 % vs. 50 - 30 % obtained by the traditional method (PI). PII has also improved chitosan yields with 37.7 - 44.3 mg/g compared to PI's 24.4 - 33.3 mg/g. This is thought to stem from the freeze-thawing cycles in PII, which have helped in preparing the chitin polymer before the deacetylation stage. Specifically, freezing and thawing the chitin polymer can help in greatly transforming the chitin polymers' texture as well as other physicochemical properties. The formed ice crystals disorganize its intramolecular and intermolecular hydrogen bonds, which in turn form a less condensed structure that is more prone to react in its upcoming deacetylation reaction. The whiteness index had also increased when using protocol PII, and

rose to 77-80 compared to 74 -77 for protocol PI. Utilizing a freeze-thawing protocol thus enables a general enhancement in chitosan quality and yield, without having to increase the use in alkali reagents or temperature. The advantages of the freeze-thawing protocol are supposedly credited to chitin de-structuration. This insoluble polymer can hardly be modified since its inner parts cannot be reached by the alkali reagent. The freezing process induces the formation of pores that open the polymer's crystalline structure, making its inner parts more accessible to the alkaline solution. This leads to a more effective deacetylation process, forming higher quality chitosan (H.P.S et al., 2016).

3.2. Characterization of fungal chitosan prepared by the PII protocol

Fungal chitosan samples were prepared from three mushrooms types using the PII protocol, and then characterized and compared to commercial chitosan that originated from crustaceans. FTIR spectra for mushroom sourced chitosan samples were found to be similar to each other and to that of their commercial equivalent. Characteristic amine absorption bands were observed at ~1600 cm^{-1}, and C-H stretch bands at ~2900 cm^{-1}, with broad bands also at 3500-3100 cm^{-1} attributed to N-H and OH-O stretch vibrations. In addition, ^{1}H NMR measurements were performed for the different-sourced chitosan polymers, and revealed similar spectra for all of the examined types with a characteristic peak at 3.15 - 3.17 ppm for C2's deacetylated **H**-C-N proton. Monomers that did not undergo deacetylation revealed their *N*-acetyl's methyl group (C-N-CO-C**H**$_3$**)** at 2.05 ppm (Figures 1S and 2S). Interestingly, all of the fungal-sourced chitosan samples had lower nitrogen contents than the animal-sourced chitosan, with 4.20, 4.25, and 3.35 % DM for white *A. bisporus, P. ostreatus*, and brown *A. bisporus,* respectively and 6.7 % DM for the commercial sample.

Thermogravimetric analyses were performed for all chitosan samples at identical conditions, yielding similar weight loss spectra patterns (Figure 1). The fungal-sourced samples are clearly reminiscent of the commercial chitosan profile, suggesting a successful polymer extraction from the mushrooms. However, specific changes were acknowledged. *P. ostreatus* chitosan decomposition set in at a lower temperature than other samples (190 vs. 220 °C). The commercial chitosan has shown to lose the most water during the heating process, and therefore had contained the greatest amount of water absorbed in it and on its surface, prior to heating. The spectrum derivative for white *A. bisporus* has displayed the least homogeneous transitions, suggesting it contains two different polymeric structures. The spectrum for the commercial sample has shown it lost all of its weight at 520 °C, while the mushroom-sourced

samples still showed weight residues at this temperature range, suggesting they commonly have different polymorphs with stronger bonds than the commercial chitosan.

The three different fungal-sourced chitosan samples were further characterized for their molecular weights, polydispersity indices, viscosity, solubility and hydrophobicity, and compared with the commercial crustacean-sourced chitosan (Table 2). Chitosan properties were found to be different among the inspected samples, with no reliance on their source of origin. Among them, the brown *A. bisporus* sample had displayed the highest molecular weight and polydispersity index (263,000 Da and 6.20, respectively). The polydispersity index for the other studied samples was found to be 2.13 - 3.66, which indicated a narrow dispersity with an overall more similar molecular weight distribution (10,600 – 31,800 Da).

Our study has also found a correlation between the intrinsic viscosity and molecular weights for the fungal-sourced samples, as previously investigated and reported (Kasaai, Arul, Charlet, 2000). Specifically, the brown *A. bisporus* -sourced chitosan sample has demonstrated the highest viscosity (406 mL g^{-1}) and molecular weight, followed by white *A. bisporus*- and *P. ostreatus*-sourced samples (343 and 232 mL g^{-1}, respectively). The commercial crustacean-sourced chitosan sample did not follow this trend, with an average molecular weight of 31,859 gr/mol and the highest viscosity value out of all the inspected samples (454 mL g^{-1}).

On the other hand, a clear correlation was found between the samples' % DA and solubility. Crustacean, brown *A. bisporus* and *P. ostreatus*-sourced samples have all showed a low % DA (6.91, 17.24 and 14.84 %, respectively), with a matching high solubility (9.06, 9.45 and 8.82 g L^{-1}, respectively). White *A. bisporus*-sourced samples on the other hand have demonstrated the highest % DA (27.71 %) with a matching solubility (7.51 g L^{-1}). This in turn makes the chosen deacetylation method a determining factor in chitosan's eventual solubility (Abdel-Gawad et al., 2017). Additional studies have shown that the molecular weight and % DA are collectively responsible for chitosan's solubility with an intermolecular force conditioned from the deacetylation of random acetyl groups (Agnihotri et al., 2004; Muzzarelli et al., 2012; Chang et al., 2015).

Chitosan samples were also inspected as solid films in order to compare their hydrophobicity and surface energy by using water contact angle measurements. While commercial chitosan had an initial water contact angle of 102.38 °, white *A. bisporus* chitosan displayed a near similar value of 101.55 °. A lower value was obtained by brown *A. bisporus* (84.73 °), demonstrating a higher surface free energy and a more hydrophilic profile that exhibits better adhesiveness and wettability.

Interestingly, among the three mushroom-sourced polymers, the brown *A. bisporus* showed the highest whiteness and lightness (Table 3). This mushroom's natural brown color did not affect its produced chitosan in terms of color and lightness. Overall, mushroom-sourced samples have demonstrated higher lightness and whiteness values when compared to their commercial animal-sourced counterpart. These observations were still evident even after subjecting these polymers to high temperatures during the TGA investigation.

X-ray diffraction (XRD) studies have demonstrated different patterns for the fungal-sourced and commercial chitosan samples. The commercial sample's characteristic broad peak at ~ 20 ° was observed in brown and white *A. bisporus*-sourced samples, yet was not observed in the *P. ostreatus*-sourced sample. On the other hand, all three fungal-sourced samples have demonstrated a repeating pattern with unique peaks at 31, 45, 56, 66, 75, and 84 ° that were not present in the commercial chitosan (Figure 2).

3.3. Chitosan antimicrobial activity

Growth curves for *B. subtilis*, *E. coli* and *S. cerevisiae* as affected by different concentrations of the brown *A. bisporus*, white *A. bisporus* and *P. ostreatus*-sourced samples were measured and compared to the commercial chitosan. The log stage was observed to be delayed to different extents due to chitosan addition. All chitosan samples exerted a microbiological inhibitory effect in the range of the tested concentrations, although their potency varied depending on the sample origin and microbial species (Figure 3).

All three types of mushroom-sourced chitosan samples have shown MIC values of 0.31 and 0.63 mg mL^{-1} against *B. subtilis* and *E. coli*, respectively, while the commercial sample's value was 0.16 mg mL^{-1} for both bacterial species (Table 4). These values were within the inspected range as reported in the literature against *Bacillus* (0.05 to 1 mg mL^{-1}) and *E. coli* (0.02 to 1 mg mL^{-1}) species (No, Park, Lee, Hwang & Meyers, 2002). For both bacterial species tested, the MBC values for all chitosan samples were equal to their MIC values (Table 4), indicating chitosan's bactericidal effect. Our observation that mushroom-sourced chitosan showed higher efficacy against *B. subtilis* than against *E. coli* agrees with results from No et al. (2002), who compared chitosan activity on Gram-positive and Gram-negative bacteria. Zheng & Zhu (2003) have suggested that the antibacterial effect mechanisms were different for Gram-positive and Gram-negative bacteria. According to their study, chitosan deposited on the surface of a Gram-positive *Streptococcus aureus* has inhibited nutrients from entering the cell, while in the case of Gram-negative *E. coli*, low molecular weight chitosan has entered the cell through a pervasion process. Furthermore, Chung & Chen (2008)

have suggested that the inactivation of *E. coli* by chitosan included an initial separation of the cell wall from its cell membrane, followed by membrane destruction.

S. cerevisiae yeast have shown in our trials a lower sensitivity to chitosan when compared to bacteria. MIC values for *S. cerevisiae* were 1.25, 0.63 and 0.31 mg mL^{-1} for Brown *A. bisporus*, White *A. bisporus*, and *P. ostreatus*, and respectively. Rhoades & Roller (2000) have found that *S. cerevisiae* 3085 was totally inhibited by 0.2 mg mL^{-1} of native chitosan, while Elmaci et al. reported chitosan's MIC against *S. cerevisiae* exceeds 2 mg mL^{-1} (Elmaci et al., 2015). Gil et al. (2004) reported that 0.1 g L^{-1} chitosan selectively inhibited bacterial growth without altering yeast viability, while up to 1 g L^{-1} of chitosan exhibited an activity against commercial brewing yeasts. In contrast to chitosan's bactericidal effect, MIC concentrations against *S. cerevisiae* were only fungistatic and allowed yeast recovery after a transfer to a fresh medium without chitosan. The fungicidal effect (MBC) was reached only with chitosan concentrations that exceeded the MIC by a two- or even a four-fold.

Understanding chitosan's exact mechanism of action against yeast is still incomplete. Savard et al. (2002) reported that *Saccharomyces unisporus* treated with chitosan-lactate with a polymerization degree of 25 has shown an agglutination of the refractive substance on the entire cell wall, suggesting its interaction with chitosan. Our results are in the agreement with the previous studies showing that chitosan with lowest % DA and lowest molecular weight had the most potent antimicrobial effect. Lower % DA is associated with positive charge and more free amino groups, making them the determinants of its antimicrobial effect (Andres, Giraud, Gerente & Le Cloirec, 2007). In addition, antimicrobial activity of chitosan depends on its solubility that are influenced by molecular weight and % DA (Omura et al., 2003). Furthermore, the improvements in preparation methods for mushroom-sourced chitosan will concomitantly enhance their antimicrobial efficacy.

4. Conclusion

A general enhancement of fungal-sourced chitosan yield and quality was achieved without amplifying alkali reagent concentration or temperature, but by using a new protocol that involves freeze-thawing cycles. The prepared chitosan samples have demonstrated an antimicrobial activity against *B. subtilis, E. coli* bacterium and *S. cerevisiae* yeast, as well as satisfactory chemical and physical parameters. Being developed for the production of non-animal fungal chitosan that is highly desired in the food industry, the described method can also be used for the production of chitosan from non-mushroom sources. Moreover, the safe

freeze-thawing technique that significantly increases polymer materials' reactivity could be utilized for modifying other edible hydrocolloids.

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Figure Captions

Figure 1**.** TGA spectra for commercial crustacean-sourced chitosan, white *A. bisporus* chitosan, *P. ostreatus* chitosan and brown *A. bisporus* chitosan (A), and their respective derivatives (B).

Figure 2**.** XRD patterns of commercial chitosan (blue), *A. bisporus* (orange), *P. ostreatus* (gray), and brown mushroon sourced chitosan (yellow).

Figure 3. Representative growth curves of *B. subtilis*, *E. coli*, and *S. cerevisiae* in the presence of specified concentrations of crustacean-sourced chitosan white *A. bisporus* chitosan, *P. ostreatus* chitosan (DA%, MW) and brown *A. bisporus* chitosan.

Tables

Table 1

Comparison of Yield, Whiteness index and Degree of acetylation (% DA) for the three mushroom sourced chitosans produced by either Protocol I and Protocol II.

	brown *A. bisporus*		
	Yield (mg g^{-1})	Whiteness index	DA (%)
Protocol I	31.8±4.2b*	77.40±1.16a	35.89±5.39b
Protocol II	42.3±0.7a	80.04±1.62a	17.24±1.44a
	white *A. bisporus*		
	Yield (mg g^{-1})	Whiteness index	DA (%)
Protocol I	33.3±2.1b	74.18±2.15a	48.13±4.23b
Protocol II	37.7±1.8a	77.38±0.52a	27.71±3.69a
	Pleurotus ostreatus		
	Yield (mg g^{-1})	Whiteness index	DA (%)
Protocol I	24.4±4.5b	75.67±0.10b	44.11±4.27b
Protocol II	44.3±3.4a	80.28±0.73a	14.84±1.55a

* Each value is expressed as mean ± S.D. (n=3). Means with different letters within a column are significantly different (P<0.05).

Table 2

Average molecular weights (Mw) [Da], molecular weight average numbers (Mn) [Da], peak molecular weights (Mp) [Da], and polydispersity indices (PDI) for all the inspected chitosan samples.

Chitosan source	Mw/ (Da)	Mn/ (Da)	Mp/ (Da)	PDI Mw/Mn	[η] (mL g^{-1})	Water contact angle (°)	Solubility** (g L^{-1})
crustacean-sourced	59,826	16,353	31,859	3.66	454	102.38 ± 1.47	9.06 ± 0.930a
brown *A. bisporus*	600,858	96,937	262,982	6.2	406	84.73 ± 1.28	9.45 ± 1.46a
white *A. bisporus*	55,645	24,342	31,206	2.29	343	101.55 ± 3.88	7.51 ± 0.60b
P. ostreatus	24,306	11,387	10,649	2.13	232	*	8.82 ± 0.54ab

** P. ostreatus was not examined, as it did not produce a measurable dry film.*

** Reported values are the means ± S.D. (n=3). Data with different letters in the same column are significantly different (P<0.05).

Table 3

Color attributes for all the inspected chitosan samples.

Sources of chitosan	*L* value	*a* value	*b* value	Whiteness index
crustacean-sourced	79.33±0.20c	0.86±0.06d	15.70±0.22a	74.03±0.29c
brown *A. bisporus*	83.73±1.74a	1.41±0.06c	11.46±0.40b	80.04±1.62a
white *A. bisporus*	80.72±0.53bc	1.65±0.04b	11.7±0.14b	77.38±0.52b
P. ostreatus	82.88±0.59ab	2.11±0.10a	9.56±0.43c	80.28±0.73a

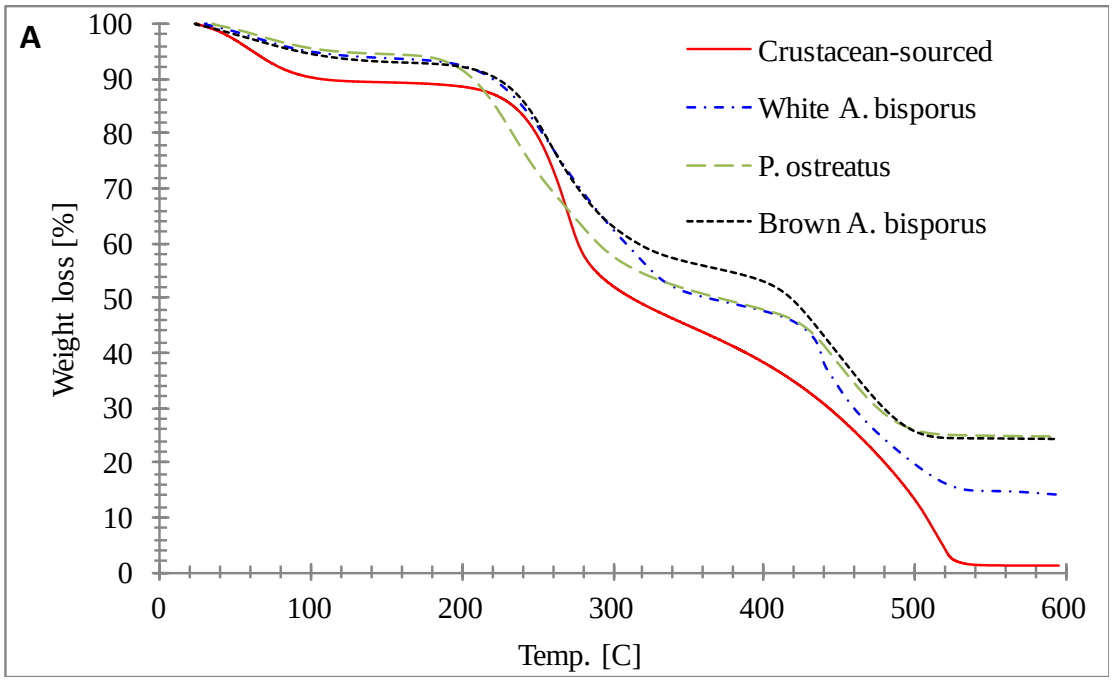
All the values were reported with the means ± S.D. (n=3). Data with different letters in the same column for each chitosan are significantly different ($P<0.05$).

Table 4

MIC and MBC values for all the inspected chitosan samples when tested against microorganisms

Species	MIC/MBC (mg mL^{-1})			
	crustacean-sourced chitosan	brown *A. bisporus chitosan*	white *A. bisporus chitosan*	*P. ostreatus chitosan*
B. subtilis	0.16/0.16	0.31/0.31	0.31/0.31	0.31/0.31
E. coli	0.16/0.16	0.63/0.63	0.63/0.63	0.63/0.63
S. cerevisiae	0.31/0.63	1.25/5.00	0.63/1.25	0.63/1.25

Figure 1



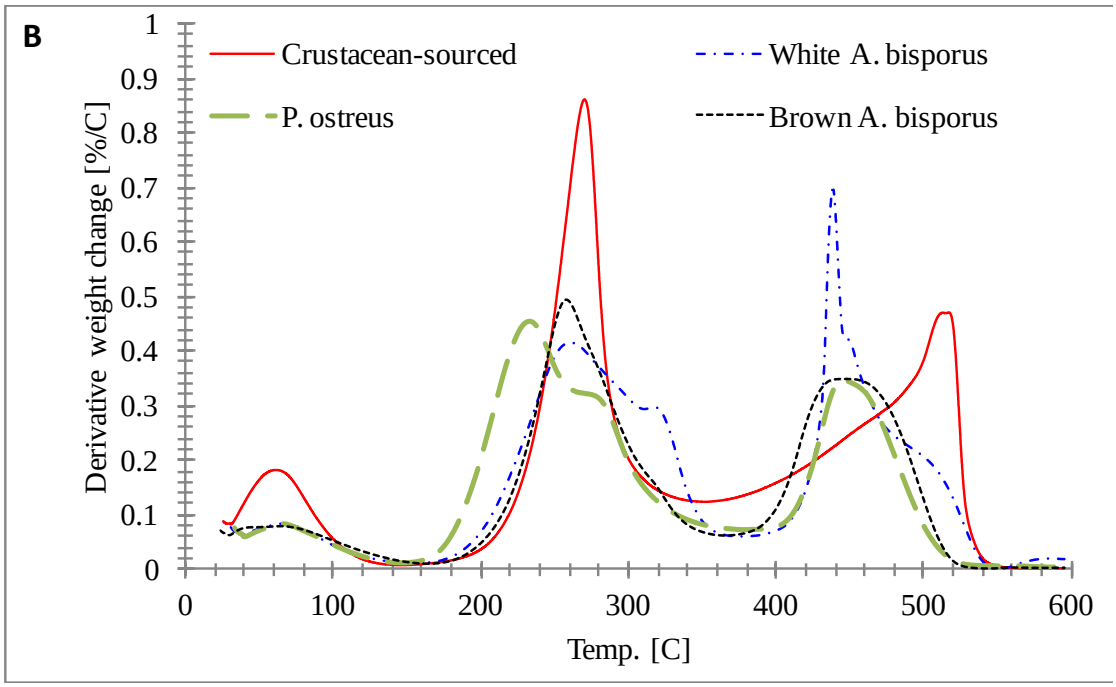


Figure 2

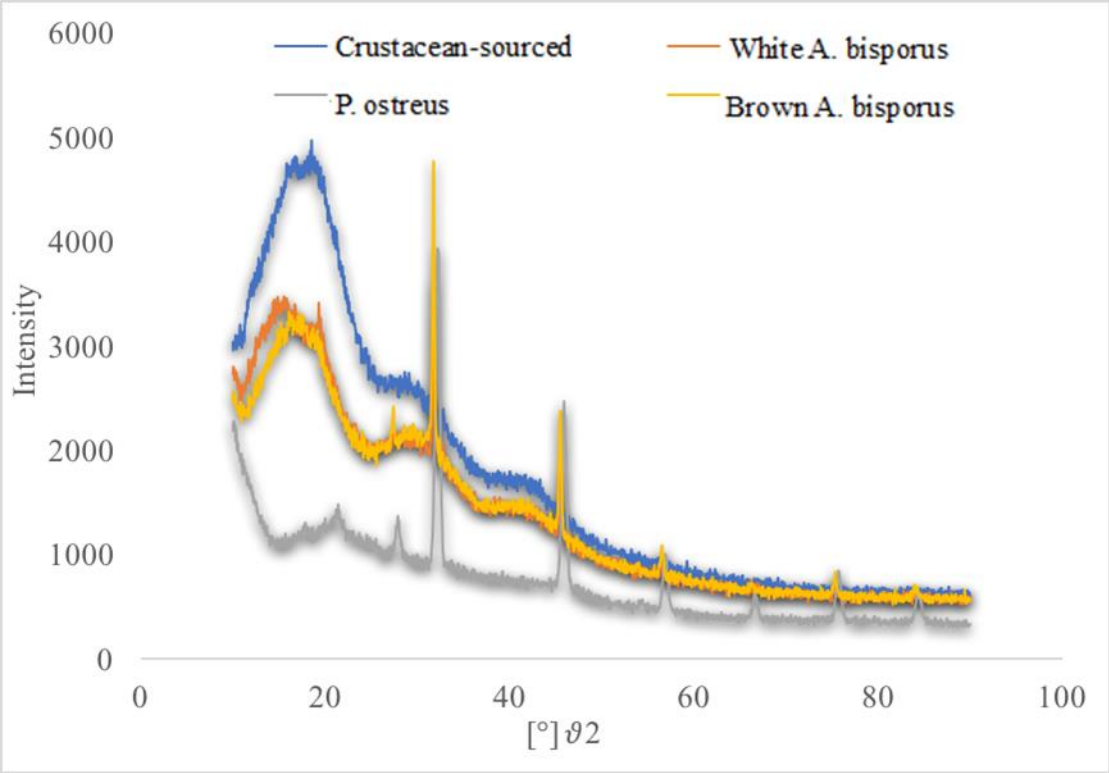
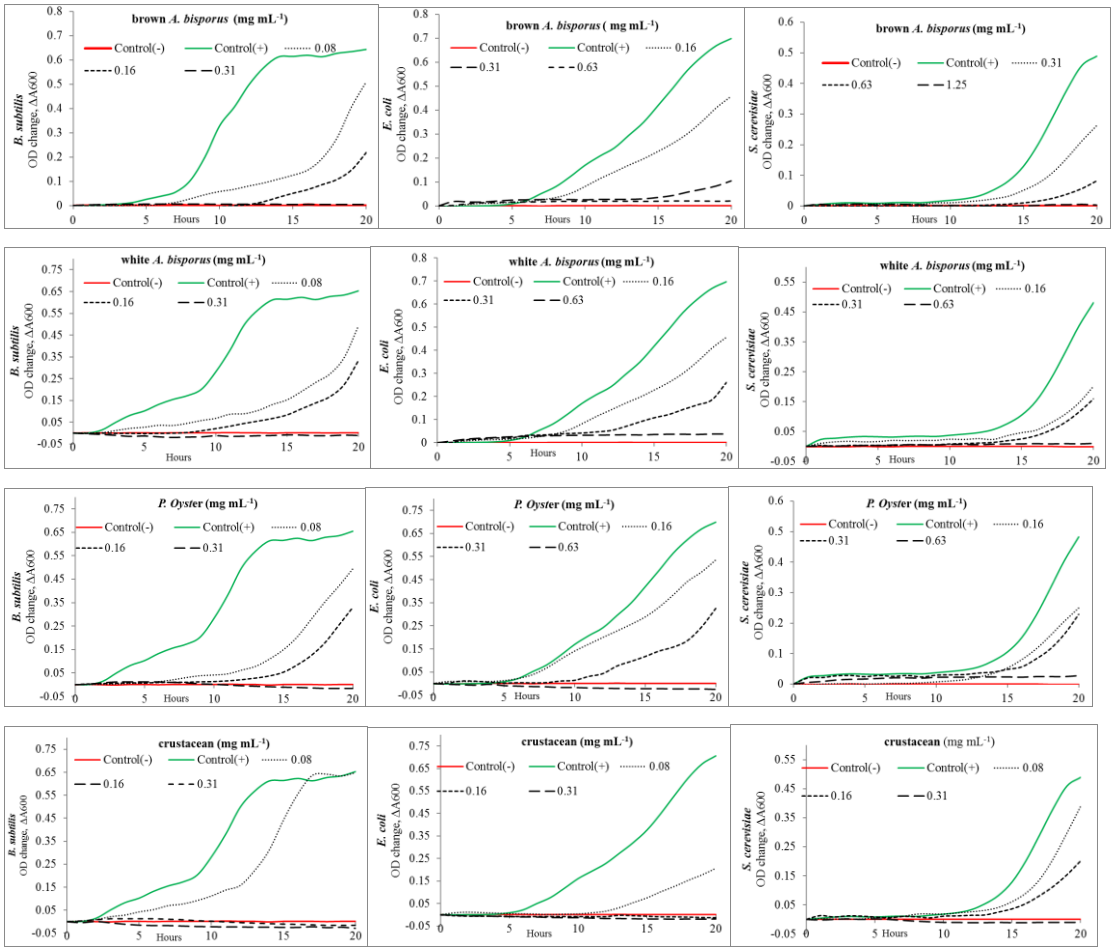

Crustacean-sourced
White A. bisporus
P. ostreus
Brown A. bisporus
Intensity
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
[°] ϑ2

Figure 3


brown A. bisporus (mg mL-1)
Control(-)
Control(+)
0.08
0.16
0.31
0.63
1.25
B. subtilis
E. coli
S. cerevisiae
OD change, ΔA600
Hours
white A. bisporus (mg mL-1)
P. Oyster (mg mL-1)
crustacean (mg mL-1)

Paper 3

Submitted to Food Chemistry

Using chitosan from mushroom waste for preservation of agricultural food products; quality and storability enhancement of fresh-cut melons

Elena Poverenov,[a]* Yana Zaicev,[a,b] Viki Bar,[a] Ofer Danay,[c] Batia Horev,[a] Cristina Bilbao-Sainz,[d] Tara McHugh,[d] Victor Rodov.[a]

[a] Postharvest and Food Science Institute. Agricultural Research Organization, The Volcani Center. Rishon Lezion, 7505101, Israel.

[b] Institute of Biochemistry, The Hebrew University of Jerusalem, Rehovot 76100, Israel.

[c] MIGAL Galilee Research Institute LTD, Kiryat Shemona 11016, Israel.

[d] Western Regional Research Center, United States Department of Agriculture, Agricultural Research Service, Albany, CA, 94710, USA.

[*]Correspondence Postharvest and Food Science Institute, Agricultural Research Organization, the Volcani Center, Rishon LeZion, 7505101, Israel. Phone: (+) 972-50-6220070, Fax: (+) 972-3-9683692, E-mail: elenap@volcani.agri.gov.il

Abstract

In this work, the possibility of usage mushroom industry wastage, as a source of antimicrobial biopolymer chitosan to form active edible coatings was studied. Physicochemical characteristics of the received fungal chitosan were examined and compared to those of the commercial, crustacean-sourced chitosan. An antimicrobial activity of the prepared chitosan was tested on *E. coli* bacteria and *Saccharomyces cerevisiae* yeast, and found to be similar to that of the commercial one. The fungal chitosans-based edible coatings we applied on fresh-cut melons were found to significantly inhibit physiological and microbial deterioration of the fruits, thereby reducing the bacteria, yeast and mold counts (up to 4 log CFU g^{-1}) and slowing down tissue texture degradation. Producing chitosan from champignon stipe offcuts will allow utilization of mushroom industry waste as a sustainable source of non-animal chitosan and promoting application of chitosan-based edible coatings as safe and effective method for preservation of fresh food products.

Keywords: Fungal chitosan; edible coating; mushroom industry waste; antimicrobial; melons.

1. Introduction

Fresh agricultural produce need approaches that allow prolonging their storability and reducing microbial decay (Ramos et al., 2013; Qadri et al., 2015; Dhall, 2013; Valencia-Chamorro et al., 2011). Chitosan-based edible coatings have been reported to effectively extend quality and storability of food products in general and of fresh agricultural produce in particular (Ghidelli and Pérez-Gago, 2017; Elsabee & Abdou, 2013; Campos et al., 2011).

Chitosan is derived from the natural polysaccharide chitin, and has unique antimicrobial properties and extensively utilized in food and biomedical applications (Rabea et al., 2003; Elsabee & Abdou, 2013). Practically all commercial chitosan is currently sourced from crustacean shellfish, i.e. shrimps and crabs. Fruits and vegetables coated by such animal-sourced chitosan become inconsumable for a considerable part of the population that include vegetarians and people who do not eat crustaceans because of allergy, religious or other restrictions. These considerations limit chitosan coatings usage in food industry, especially in the field of fresh agricultural produce. Therefore, the alternative non-animal source that would allow wide application of chitosan as edible coatings is needed.

Cell wall of mushrooms includes chitin fibers and could became a non-animal source for chitosan production (Kalac, 2013). Several reports described production of chitosan from various mushrooms (Pochanavanich, 2002; Wu, 2004; Yen, 2007; Bilbao-Sainz et al., 2017). Moreover, chitosan production may not require high quality mushroom and can utilize waste of mushrooms industry. In most mushroom processing factories, the cut-off stipe edges are not fully utilized and treated as a waste (Zhang et al., 2012).

In this work, the possibility of usage mushroom stipe offcuts, wastage of mushroom industry, as a chitosan source to form active edible coatings was studied. Yield and quality of the chitosan received from champignon stipe and cap were compared. Then, fungal chitosan from both sources was compared to a commercial animal-sourced chitosan in terms of structure and antimicrobial activity. Finally, fungal and animal-sourced chitosan were applied as edible coatings on fresh-cut melons and their effect on physiological and microbial quality and the storability of the fruits was studied.

2. Materials and methods

2.1 Plant material

Fresh cut mushrooms strain A15 (Sylvan USA) and mushrooms stipe offcuts were collected from a Champignon Farm in Zarit, Israel. The mushrooms products were kept at 3^{o} C until the production of the fugal chitosan.

Melons (*Cucumis melo* var. *cantalupensis*, cv. Yaniv) were purchased from Ein Yahav farm (Northern Arava, Israel). Whole melons were decontaminated with 200 ppm solution of sodium hypochlorite, rinsed with water and air-dried. Pulp plugs from twenty-eight melons different melons were excised from the "equator" region using a sterile cork borer (1.7 cm diameter and length 4 cm). The 560 plugs were mixed to receive a random distribution of fruit samples.

2.2 Production of fugal chitosan

Champignon chitosan was prepared utilizing combination and modification of the reported literature procedures (Wu, 2004; Yen, 2007). (A) One liter of 2M NaOH was added to 100 g mushroom caps or stipes and the mixture was heated upon gentle stirring for 2 h at 100 °C. The alkali insoluble phase was collected, washed with double distilled water and heated in 1 % w/v oxalic acid solution (1 L) for additional 1 h at 100 °C. The mixture was cooled to room temperature and an insoluble phase was collected, washed with DDW and lyophilized. (B) Aqueous solution of 10 M NaOH was added to the received dry material (30 mL per 1 g of dry solid) and the mixture was heated at 100 °C for 2 h. The mixture was cooled to room temperature and a solid phase was separated by centrifugation (9000 rpm for 40 min), washed with DDW until receiving pH of 5.7 and dried by lyophilization. (C) Aqueous solution of 2% v/v acetic acid was added to the received dry material, 40 mL per 1 g of dry solid, and the mixture was refluxed at 90 °C for 2 h. The mixture was cooled to room temperature and a liquid phase was separated by decantation. (D) NaOH solution (2M) was added until receiving pH 10. The precipitated chitosan was collected by centrifugation (12000 rpm for 15 min), washed with DDW and dried by lyophilization. The procedure was repeated three times and average chitosan yield of the three repetitions was expressed as mg/g, mg of chitosan per 1g of dry mushroom crude.

2.3 Characterization of fungal chitosan

FTIR spectra of the chitosan powder mixed with anhydrous KBr (KBr/material mass ratio 1 : 100) were recorded between 400 and 4000 cm^{-1} with 100 scans averaged with a resolution of 4 cm^{-1} (Bruker Tensor 27 FTIR Spectrometer). The absorption measurements of the prepared films were performed by Jenway 6505 UV/vis Spectrophotometer.

2.4 Antimicrobial activity evaluation

Cells of yeast *Saccharomyces cerevisiae* (YSC2, Type II, Sigma) were grown overnight in nutrient yeast dextrose broth (NYDB) liquid medium, separated from the medium by centrifugation, washed with sterile water and resuspended in a fresh double-strength NYDB medium (pH 5) to reach optical density of 0.2 at a wavelength of 600 nm. The yeast suspensions were further diluted 1:1 with aqueous chitosan solutions and sterile water acidified with acetic acid to pH 5 to obtain various concentrations of chitosan but the same initial *S. cerevisiae* dilution. The yeast suspensions were aseptically pipetted into wells of 96-well microplate, 0.2 mL per plate and incubated for 12 h at 27 °C in EnSpire multilabel plate reader (Perkin Elmer, Waltham MA, USA) with hourly 10 s shaking. In addition to treatments (nutrient medium with viable *S. cerevisiae* + chitosan), the experiments included blanks (nutrient medium without *S. cerevisiae* + chitosan), positive controls (nutrient medium with viable *S. cerevisiae*, without chitosan) and negative controls (nutrient medium with dead *S. cerevisiae* killed by boiling + chitosan). The yeast growth was examined by hourly measurement of turbidity as optical density at 600 nm and expressed as turbidity difference (ΔA_{600}) between the samples with viable yeast cells and the corresponding negative controls. The experiments were run in triplicate with commercial and fungal chitosan samples in final concentrations of 0.5, 1.0 and 2.0%. Similar technique was used for evaluating the effect of chitosan on bacterium *Escherichia coli* (ATCC 25922) growth, except for using the LB broth (Lennox) instead of the NYDB medium.

2.5 Preparation and application of edible coating solutions

Edible coating were prepared by dissolving at room temperature 1.5g of chitosan powder in 100 ml of double distilled water (DDW) that contained 0.7 ml of acetic acid. Melon plugs were immersed in the chitosan solution for 30 Sec and then dried under sterile air flow for 30 min at room temperature. Four treatments including melon plugs coated with (a) stipe sourced fungal chitosan, (b) cap sourced fungal chitosan, (c) commercial crabs sourced chitosan (Sigma Aldrich, Israel) and (d) uncoated melons as a control have been performed. Each included treatment was performed in triplicate. The melon plugs were stored at 6 °C in PETE clamshell containers as a simulation of cooled retail display conditions.

2.6 Microbiological analyses

4 weighed melon plugs (~ 20 g) were transferred into sterile Stomacher bag that contained 180 ml of sterile 0.9% NaCl solution and homogenized for 3 min in a Stomacher 400 circulator (Seward, Worthing, UK). Test samples were serially diluted in saline solution. Aerobic plate counts were determined by surface inoculation of plate count agar (PCA) (Oxoid, Basingstoke, UK) and mold and yeast counts were determined by surface inoculation of potato dextrose agar supplemented with 100 ppm chloramphenicol antibiotics (PDA+A). The PCA plates were incubated at 30 °C for 48 h and PDA+A at 25 °C for 5 d. The colony-forming units (CFU) per gram of melon material was calculated. Four samplings at 0, 5, 9 and 11 days were performed. Three replications per sampling from three containers were done. For statistical analysis, the data were transformed into logarithmic form as decimal logarithms of the CFU/gr. A total value of 0.9 CFU was assigned to all Petri dishes in a sample showing no colonies at the least dilution.

2.7 Weight loss analysis

For a weight measurement, three randomly chosen plugs were taken from three containers every two days during all storage (nine replicates for each treatment at each sampling). The weight loss is reported as

$$\text{Weight loss (\%)} = \frac{\text{weight}_{\text{day=0}} - \text{weight}_{\text{sampling}}}{\text{weight}_{\text{day=0}}} * 100\ \%$$

2.8 Texture analysis

Textural studies of the melon plugs were performed utilizing puncture tests with a TA-XT2 Texture Analyzer (Stable Micro Systems Ltd., Godalming, UK) using a 2-mm-dia stainless cylinder probe at speed of 1.0 mm s^{-1}. were done. Fifteen replicates were analyzed for each treatment (three containers containing 5 melon plugs).

2.9 Atmosphere composition

Headspace atmosphere samples were withdrawn from three replicate containers of each treatment by gas-tight syringes. Gas chromatography (Varian 3300) was utilized to quantify ethanol and acetaldehyde vapors concentrations using external standards. The gas chromatograph equipped with a Carbowax 20M packed column, flame ionization detector and a 20% and helium as the carrier gas were utilized. The injector, column, and detector temperatures were 110, 80 and 180°C, respectively. Oxybaby 6.0 gas analyzer (Witt-

Gasetechnik GmbH & Co KG, Witten, Germany) that comprises an electro-chemical cell for oxygen and an IR-absorption cell for CO_2 analysis was used to determine carbon dioxide and oxygen concentrations.

2.10 Statistical Analysis

Microsoft Office Excel spreadsheets were used to calculate means, standard deviations, and 95% t confidence intervals. The statistical analyses were carried out using JMP Version 5.0.1 software (SAS Institute Inc., Cary, NC, USA) including a one-way analysis of variance (ANOVA) followed by the Tukey honestly significant difference (HSD) post-hoc test.

3. Results & discussion

3. 1 Production and characterization of fungal chitosan

Fungal chitosan was yielded from champignon mushroom caps or stipe offcuts. Interestingly, champignon stipe yielded 176 mg chitosan per 1 g dry mushroom source, higher than the champignon caps (105 mg g^{-1} dry matter). This observation is of practical importance. Mushrooms stipe are usually underutilized part of the mushroom that is wasted in many tons by mushroom industry (Danay et. al., 2012). Producing chitosan from champignon stipe offcuts will allow utilization of mushroom industry waste bringing both economic and environmental benefit.

Fungal chitosan from both cap and stipe sources were characterized utilizing FTIR spectroscopy. FTIR spectra of the fungal chitosan was found to be similar to that of the commercial one (Duan, 2004) and demonstrated characteristic amine absorption bands around 1600 cm^{-1}, C-H stretch bands around 2900cm^{-1} and broad bands at 3500-3100 cm^{-1} attributed to N-H and OH-O stretching (Fig. 1).

3. 2 In vitro antimicrobial activity of fungal chitosan

The antimicrobial effect of the fungal and commercial chitosan samples was examined on yeast and bacteria strains. Yeasts, including *S. cerevisiae*, are known as one of the major spoilage causes in fresh-cut fruits and other fruit products e.g. juices (Loureiro and Querol, 1999; Restuccia et al., 2006; Krisch et al., 2016). Aqueous 0.5% solution of commercial chitosan resulted in total inhibition of *S. cerevisiae* growth (Fig. 2). Fungal chitosan caused significant inhibition of yeast growth at 0.5% and completely prevented the growth of *S. cerevisiae* at 1 and 2%. All the examined chitosan solutions demonstrated significant inhibition of *E. coli* growth (Fig. 3). Upon treatment with fungal chitosan solutions of 0.5 and 1.0%, mild bacterial proliferation was observed. The 2.0% solution of fungal chitosan, as well as 0.5% commercial chitosan solution, caused total bacteria growth inhibition.

3.3 Fungal chitosan based edible coating of fresh cut melons

Melons are among the most commercially important fresh-cut fruit products that represent about 22% of the market (Cook, 2011) and they contain many essential nutrients such as potassium, vitamins A and C, naturally low in fat and sodium and are popular because of their unique flavor (Lester, 1997). However, fresh-cut melons are prone to fast physiological and microbial deterioration such as softening, juice leakage, flavor degradation, weight loss and microbial spoilage (Ferrari et al., 2011; Fallik et al., 2005).

Antimicrobial Protection

Natural microbial populations total aerobic counts (PCA) molds and yeast counts (PDA+A) of fresh-cut melons were studied (Table 1). After 5 days of storage, the total aerobic counts of the non-coated melons reached 7.98 log CFU/g while the counts of the melons coated with chitosan from mushroom caps and stipes were 4.05 and 6.33 log CFU/g, respectively, demonstrating the antimicrobial effect of fungal chitosan. On the same day, the total aerobic counts of the commercial chitosan-coated melons were 5.40 log CFU/g. The significant inhibition of microbial growth on chitosan-coated melons, as compared to the uncoated ones, was maintained throughout the storage period.

The coatings effect on mold and yeast growth was most dramatic after 9 days of storage. Then, mold and yeast count of non-coated melons were 4.12 log CFU/g, while counts of the chitosan-coated melons were found to be 1.22 (caps), 1.90 (stipe) and 3.26 log CFU/g (commercial). After 11 days of storage all chitosan-based coatings reduced the mold and yeast counts by more than one order of magnitude as compared to the non-coated melons.

Antimicrobial activity is one of the most required and desired contributions of edible coating to food safety and quality (Campos et al., 2011). Using animal-sourced chitosan for coating was reported to control the growth of spoilage and pathogenic microorganisms on fresh-cut melon (Sangsuwan et al., 2008). This study has demonstrated that the edible coating prepared from fungal chitosan is not inferior to the commercial chitosan in antimicrobial protection ability and in several cases even exceeded it.

Total aerobic count of 7 log CFU g^{-1} is often considered a threshold of acceptability of fresh-cut fruits and vegetables (Cefola et al., 2014) including melons (Botondi et al., 2016). On the other hand, the maximal permissible level of molds and yeasts in ready-to-eat fresh-cut produce can be set as 2.7 log CFU g^{-1} (based on the Israeli standard limits SII, 2006). According to these thresholds, coating fresh-cut melons with fungal chitosan extended their shelf life at 6 °C from <5 to 11 days based on total aerobic count and from 5 to 9 days based on mold and yeast count.

Firmness and weight loss

Examination of firmness of coated and uncoated fresh-cut melons demonstrated a clear beneficial effect of chitosan coatings. Fig. 4 presents texture patterns of the coated and non-coated melons after 9 days of storage. It can be seen that chitosan coated melon pieces had firmer texture than the uncoated ones. It is well established that edible coatings physically enhance the structure of fresh-cut produce and slow down their texture degradation (Baldwin et al., 2011). The animal sourced chitosan coating was reported to protect firmness of fresh cut melons (Poverenov et al., 2014). The fungal chitosan coating, especially stipe sourced one, also demonstrated good texture protection abilities in terms of fruit firmness. On the other hand, no effect of coating was observed on inhibition of fruit weight loss (data not shown). Such performance, effective structure

enhancement with no significant inhibition of weight loss, has been previously reported for coatings based on chitosan and other hydrophilic natural polysaccharides (Arnon et al., 2014).

Atmosphere composition

Significant problem faced upon application of edible coatings to fresh products is formation of hypoxic conditions indicated by generation of off-flavor volatiles and enhanced CO_2 production (Han & Gennadios, 2005). Headspace atmosphere composition of the coated and the uncoated melons is presented in Table 2. No increased CO_2 concentration upon application of edible coating was observed after 2 days of storage. After the extended storage of 7 and 14 days the CO_2 concentration in the containers with chitosan-coated melon plugs was significantly lower as compared to the containers with non-coated ones. The enhanced CO_2 accumulation upon prolonged storage of fresh-cut products is associated with microbiological factors such as spoilage bacteria and yeasts (Jacxsens et al., 2003). Therefore, the antimicrobial effect of chitosan coating resulted in the inhibition of CO_2 accumulation. Similar trend was observed with the headspace concentration of ethanol vapor, the fermentation associated volatile material. After 14 days of storage, coating with fungal chitosan resulted in significantly lower ethanol accumulation as compared with non-coated control. Similar inhibition of CO_2 and volatile fermentation markers accumulation by chitosan-based coatings was reported previously (Poverenov et. al., 2014). Spanier et al. (1998) suggested that ethanol and other low-boiling alcohols accumulated during storage of fresh-cut pineapple were derived from fermentation by native yeasts. Therefore, it seems expectable that inhibition of yeast growth on fresh-cut melons by chitosan coating resulted in reduction of ethanol production.

4. Conclusions

Antimicrobial biopolymer chitosan was produced from champignon mushrooms. Two sources of chitosan, stipe offcuts and cap, were studied. The stipe source resulted in higher yield of chitosan than cap, confirming that mushroom industry waste can be utilized as a sustainable source of non-animal chitosan. Spectroscopic and antimicrobial properties of the received fungal chitosan were studied and found to be very similar to

those of the commercial chitosan from crustacean source. When utilized as edible coating, fungal chitosan from both stipe and cap significantly inhibited physiological and microbial deterioration of fresh-cut melon. Producing chitosan from champignon stipe offcuts will allow utilization of mushroom industry waste bringing economic and environmental benefit and promoting application of chitosan-based edible coatings as a safe and effective method for preservation of fresh food products.

Acknowledgments

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Figures

Fig. 1. FTIR spectra of the fungal and commercial chitosan.

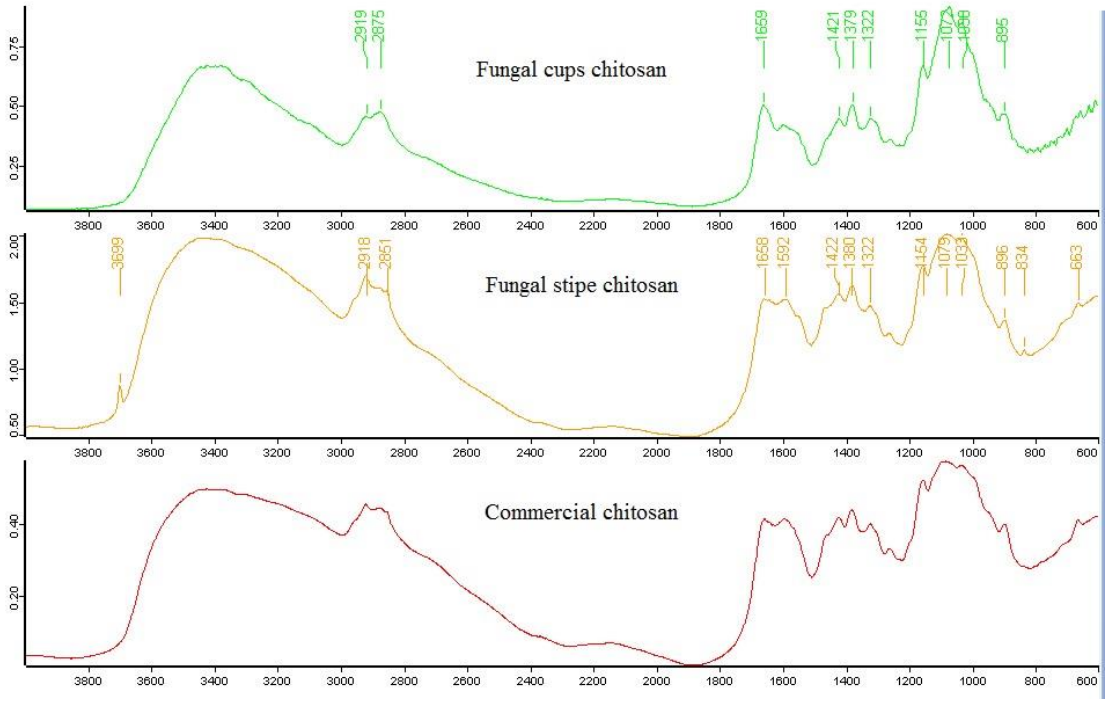


Fig. 2. Effect of chitosan source and concentration on the *Saccharomyces cerevisiae* yeast growth.

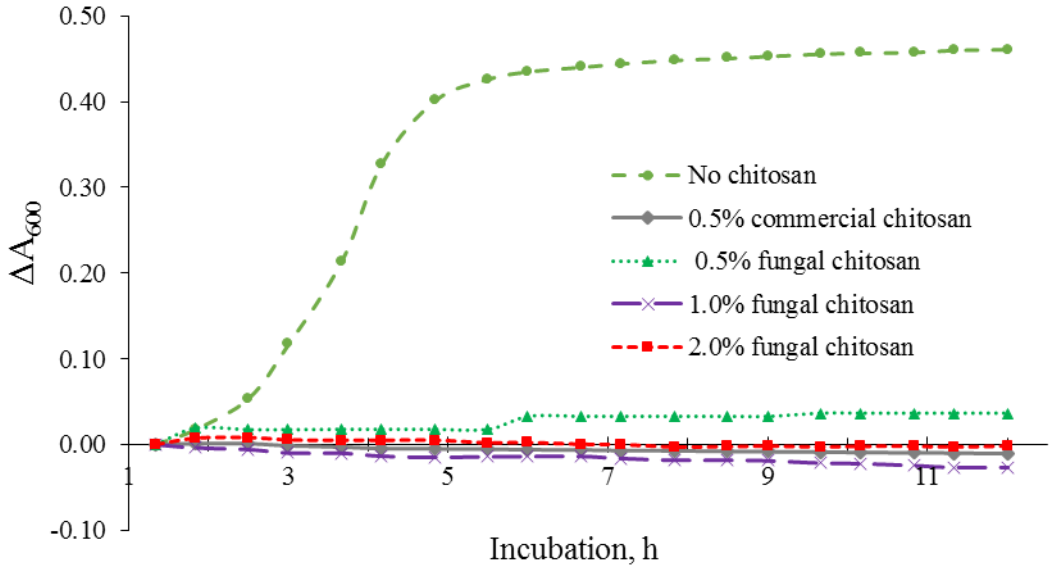


Fig. 3. Effect of chitosan source and concentration on the growth of *E. coli* bacterium.

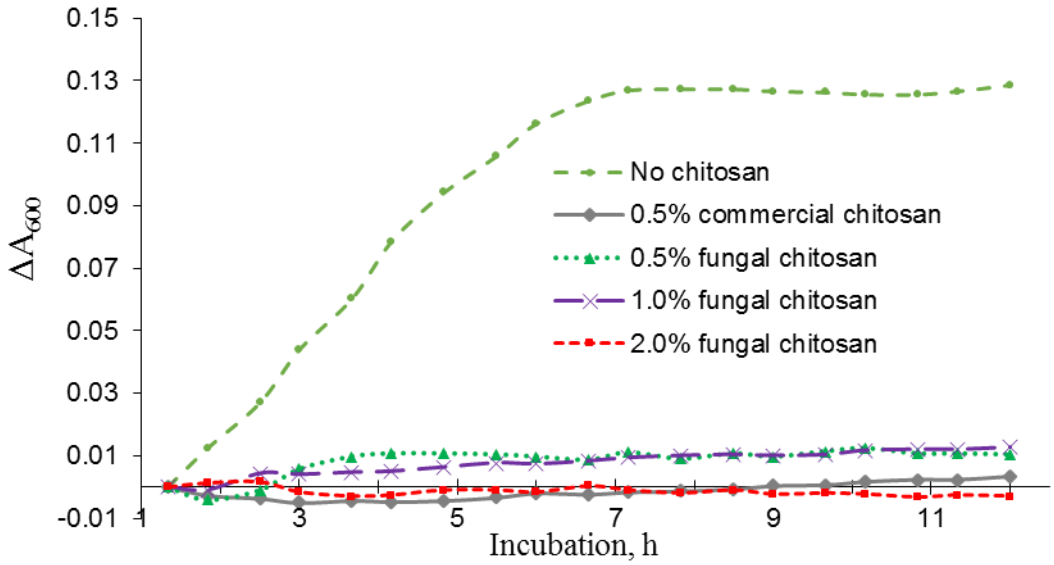
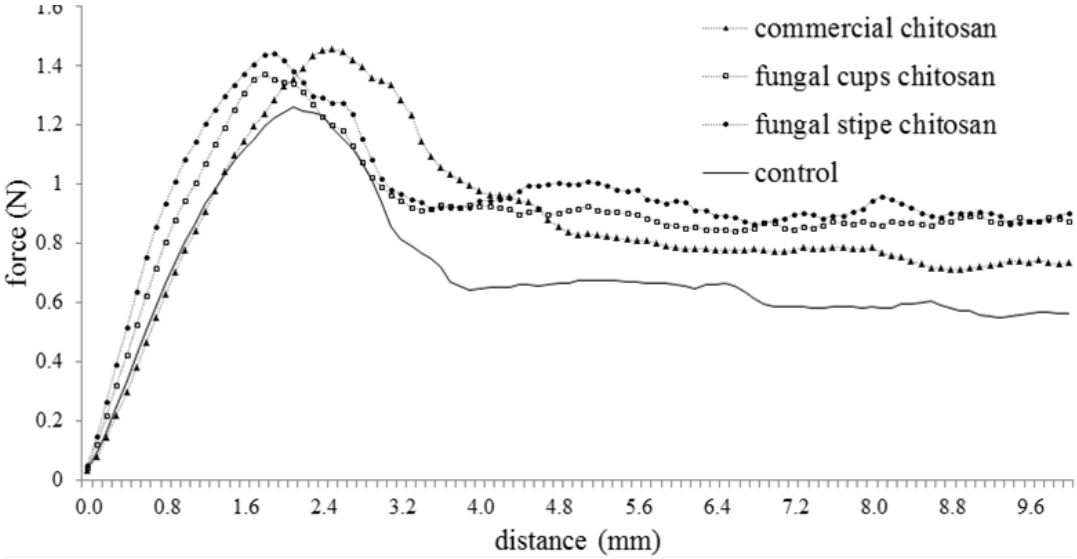


Fig. 4. Effect of coatings on fresh-cut melon texture after 9 days of storage at 6 °C. Values represent means of fifteen replications.



Tables

Table 1. Total aerobic counts and total mold and yeast counts (log CFU g^{-1}) of fresh-cut melons during storage at 6 °C. Values represent means of nine replications ± 95% t confidence intervals. The letters represent comparisons between the treatments at the same sampling time. The values followed by the same letter are not significantly different according to Tukey HSD test at $p \leq 0.05$.

PCA (log CFU/gr)	**0 days**	**5 days**	**9 days**	**11 days**
fungal chitosan- cap	1.95 ± 0.14^{a}	4.05 ± 0.29^{bc}	6.39 ± 0.40^{b}	6.92 ± 0.45^{b}
fungal chitosan- stipe	1.94 ± 0.12^{a}	6.33 ± 0.40^{ab}	6.44 ± 0.40^{b}	6.54 ± 0.23^{b}
control	2.00 ± 0.24^{a}	7.98 ± 0.45^{a}	8.95 ± 0.32^{a}	9.04 ± 0.20^{a}
commercial chitosan	2.00 ± 0.07^{a}	5.40 ± 0.45^{b}	6.85 ± 0.32^{b}	7.00 ± 0.32^{b}

PDA+A (log CFU/gr)	**0 days**	**5 days**	**9 days**	**11 days**
fungal chitosan- cap	0.69 ± 0.10^{a}	0.91 ± 0.11^{b}	1.22 ± 0.11^{c}	3.70 ± 0.05^{b}
fungal chitosan- stipe	0.8 ± 0.05^{a}	1.00 ± 0.13^{b}	1.90 ± 0.14^{c}	3.20 ± 0.20^{b}
control	0.8 ± 0.12^{a}	1.90 ± 0.20^{a}	4.12 ± 0.20^{a}	4.21 ± 0.20^{a}
commercial chitosan	0.70 ± 0.09^{a}	0.89 ± 0.08^{b}	3.26 ± 0.26^{b}	3.67 ± 0.15^{b}

Table 2. Effect of coatings on the levels of CO_2 (kPa) and ethanol (ppm) in the fruit container headspace. Each value represents means of three replications ± 95% t confidence intervals. The letters represent comparisons between the treatments at the same sampling time. The values followed by the same letter are not significantly different according to Tukey HSD test at $p \leq 0.05$.

CO_2	**1 days**	**7 days**	**14 days**
fungal chitosan cap	0.110±0.025[a]	0.392±0.048[b]	0.439±0.030[b]
fungal chitosan stipe	0.143±0.025[a]	0.446±0.046[b]	0.411±0.208[b]
control	0.264±0.092[a]	1.094±0.800[a]	4.546±1.130[a]
commercial chitosan	0.096±0.034[a]	0.115±0.013[c]	0.515±0.295[b]
Ethanol	**1 days**	**7 days**	**14 days**
fungal chitosan cap	12.80±8.03 [a]	32.45±5.65 [a]	51.36±2.61[b]
fungal chitosan stipe	32.46±11.83 [a]	32.22±3.56 [a]	48.83±1.52[b]
control	23.47±2.59 [a]	50.80±20.42 [a]	108.10±18.87[a]
commercial chitosan	13.39±8.22 [a]	39.83±3.12 [a]	78.87±1.30[ab]

Paper 4

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Layer-by-layer alginate and fungal chitosan based edible coatings applied to fruit bars

Cristina Bilbao-Sainz[a], Bor-Sen Chiou[b], Kaylin Punotai[a], Donald Olson[a], Tina Williams[b], Delilah Wood[b], Victor Rodov[c], Elena Poverenov[c], Tara McHugh[a]

[a] Healthy Processed Foods Research, U.S. Department of Agriculture, Albany, CA, United States

[b] Bioproducts Research Unit, U.S. Department of Agriculture, Albany, CA, United States

[c] Postharvest and Food Science Institute, Agricultural Research Organization, The Volcani Center, Rishon LeZion 50250, Israel.

ABSTRACT

Food waste is currently being generated at an increasing rate. One proposed solution would be to convert it to biopolymers for industrial applications. We recovered chitin from mushroom waste and converted it to chitosan to produce edible coatings. We then used layer-by-layer (LbL) electrostatic deposition of the polycation chitosan and the polyanion alginate to coat fruit bars enriched with ascorbic acid. The performance of the LbL coatings was compared with those containing single layers of fungal chitosan, animal origin chitosan and alginate. Bars containing alginate-chitosan LbL coatings showed increased ascorbic acid content, antioxidant capacity, firmness and fungal growth prevention during storage. Also, the origin of the chitosan did not affect the properties of the coatings.

Practical Application: Mushroom stalk bases could be an alternative source for isolating chitosan with similar properties to animal-based chitosan. Also, layer-by-layer assembly is a cheap, simple method that can improve the quality and safety of fruit bars.

Keywords: chitosan, mushroom, waste, layer-by-layer, edible coating

INTRODUCTION

In 2014, the consumption of whole fruits was 296 eatings per capita (eatings represent frequency of consumption but does not measure volume consumed). Fruit eatings increased by 7% between 2003 and 2014 with children under the age of 10 and adults over the age of 60 consuming the most amount. The consumption of fruits is expected to increase 5% by 2018 with a larger portion of the increase expected from early grade-school children and young adults in their mid to late 20's. (Produce for Better Health Foundation, 2015). The top reasons that affect food selection include ease of preparation, taste, whether it is filling, whether it appeals to everyone, price, and ease of planning (Produce for Better Health Foundation, 2015). Fruit bars have been developed from many types of fruits to promote healthy fruit consumption. The quality of the fruits in the bars can be preserved by coating the bars to delay moisture migration and slow O_2 transport. Edible coatings can also prevent physical damage to the bars and provide protection against external microbial contamination, extending their shelf life.

Alginate and chitosan have shown great potential for use as food coatings due to their biodegradability, biocompatibility, non-toxicity and versatile chemical and physical properties. Alginate is a polysaccharide that forms a gel by binding with divalent metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}). Chitosan is a cationic linear polysaccharide composed of D-glucosamine and, to a lesser extent, *N*-acetyl-D-glucosamine with a β-1,4-linkage (Rinaudo, 2006). Chitosan is produced by the deacetylation of chitin typically extracted from shellfish exoskeletons. However, chitosan can also be extracted from the cell wall of some microorganisms and fungi. Several mushrooms genera including *Agaricus, Pleurotus, Ganoderma* and *Lentinula* have been suggested as alternative chitosan sources (Kannan, Nesakumari, Rajarathinam, & Singh, 2010; Ospina, Ospina Alvarez, Escobar Sierra, Rojas Vahos, Zapata Ocampo, & Ossa Orozco, 2015; Yen & Mau, 2007). Moreover, chitosan can be extracted from mushroom industry waste rather than from high-quality mushrooms (Bilbao-Sainz et al., 2017). During harvesting, mushroom stalk bases are generated as a waste product. These bases comprise approximately 25% to 33% of the weight of fresh mushrooms and are normally used as low value animal feed and compost (Chou, Sheih, & Fang, 2013).

The development of new edible coatings with improved functionality and performance is one of the challenges in the food industry. Traditionally, coatings have been applied to food products as single layers; however bi-layers of different coating materials can provide more versatile control of coating properties and functionality. Layer-by-layer (LbL) assembly can be used to form multiple coating layers by alternately dipping the substrate into solutions containing oppositely charged polyelectrolytes. LbL assembly produces ultrathin polyelectrolyte multilayers on charged surfaces (Vargas, Pastor, Chiralt, McClements & González-Martínez, 2008). Chitosan, poly-L-lysine, pectin and alginate are the most commonly used biopolymers in LbL assembly (Marudova, Lang, Brownsey & Ring, 2005; Krzemiski et al., 2006; Bernabe, Peniche, Argüelles-Monal, 2005).

In this study, we formed LbL coatings on fruit bars through electrostatic interactions between negatively charged carboxylic groups of alginate and positively charged ammonium groups of chitosan. To our knowledge, the use of sodium alginate–chitosan polyelectrolyte complexes as food coatings is an innovative idea; this combination has only been applied to fresh cut melon (Poverenov, Danino, Horev, Granit, Vinokur & Rodov, 2014). In that study, the LbL coating was found to possess the beneficial properties of both materials with good adhesion of the inner alginate layer to the melon matrix along with anitimicrobial activity of the outer chitosan layer. Therefore, the objective of this study is to compare the performance of LbL alginate-chitosan coatings to those of fungal and commercial chitosan coatings on fruit bars during storage.

MATERIALS AND METHODS

Processing of fruit bars

White grape concentrate was provided by American Fruits and Flavors, LLC (Pacoima, CA) and powdered pear flakes were provided by Tree Top (Selah, WA). Fruit bars were prepared by continuously mixing white grape concentrate with powdered pear flakes at a ratio 1:1 in a food processor. L-(+)-ascorbic acid was added to the blend to provide 45 mg of AA per bar, equivalent to 100% of the recommended intake according to the World Health Organization and 50% of the recommended intake according to United States' National Academy of Sciences. The mixture was poured into aluminum

trays covered with a nonstick sheet. After flattening the mixture with a rolling pin, a mold was pressed into the mixture to cut rectangular shaped fruit bars. The bars were released from the molds and kept in the refrigerator at 5°C until further processing.

Chitosan production from mushroom stalk bases

On the day of mushroom harvest, Monterey Mushrooms, Inc. (Watsonville, CA) generously provided brown mushroom (*Agaricus bisporus*) byproducts. Mushrooms were classified by the facility as medium sized, with cap diameters typically ranging from 3 to 4.5 cm. Chitosan from mushroom stalks was produced as indicated in our previous manuscript (Bilbao-Sainz et al., 2017). Briefly, chitin from freeze dried mushroom stalk bases was extracted with 96% ethanol. Crude chitin was obtained after demineralization with sodium metabisulfite solution (1000 ml of 0.5% $Na_2S_2O_5$ dissolved in 5% HCl), deproteinization with 2% NaOH and decoloration using 0.1 M NaOH and 3% H_2O_2. Crude chitin was deacetylated using 50% sodium hydroxide.

Coating solutions and treatment

Sodium alginate from brown algae and $CaCl_2$ were supplied by Sigma-Aldrich (St. Louis, MO, USA). Sodium alginate solutions were prepared by slowly adding 1.5% (w/w) sodium alginate to distilled water. The alginate solution was brushed onto fruit bars; afterwards, 5% aqueous solution of $CaCl_2$ was brushed onto the bars' surface to perform gelation by cross-linking alginate COO^- groups with Ca^{+2}.

Low molecular weight (LMW) chitosan (m_v = 83 kDa, DD = 87%) was supplied by Sigma-Aldrich (St. Louis, MO, USA). LMW chitosan was prepared by dispersing 1.5%, (w/v) chitosan in an aqueous solution of lactic acid [0.7% (v/v)]. This solution was brushed onto fruit bars. Fungal chitosan (m_v = 21 kDa, DD = 91%) coated bars were prepared following the same procedure as that for LMW chitosan. For the preparation of alginate-chitosan LbL coatings, fruit bars were first coated with alginate and $CaCl_2$, rinsed with water and coated again either with LMW chitosan or with fungal chitosan. The different coated bars were air dried at 40 °C until the water activity reached similar levels for coated and non-coated bars. The bars were then stored in a closed chamber at room temperature and 100% RH for accelerated storage studies.

Rheology of coating solutions

The viscosity of the coating solutions was determined using a TA Instruments (New Castle, DE) AR2000 rheometer. The sample was placed in a concentric cylinder and then equilibrated at 23 °C for 5 min before the start of each experiment. The viscosity of the sample was then determined at 23 °C by varying the shear rate from 0.1 to 500 s^{-1}.

Determination of the coating thickness

The coating thickness was determined by light microscopy. Approximately 1.5 mm sections were hand-cut from the middle of each bar using a long microtome razor blade (Leica 819, low profile disposable microtome blades, Leica Biosystems, Inc., Buffalo Grove, IL). The top middle part of each section (roughly 14 mm in width) was photographed in three segments using transmitted light in a Leica MZ16F stereo microscope (Leica Biosystems, Inc., Buffalo Grove, IL) and a Retiga 2000R FAST color camera (Qimaging, Surrey, BC, Canada). Five measurements of the film thickness were taken at random locations in each photograph using ImagePro Plus 7 (Media Cybernetics, Rockville, MD).

Water Activity and Moisture Content

The water activity (a_w) was measured at 25°C by the dew point method with an AquaLab 4TE water activity meter (Decagon Devices, Pullman, WA). Moisture content of fruit bars over time was determined using a vacuum oven following the AOAC method (AOAC 2006, Method 40.1.04). The measurements were performed in triplicate for each treatment.

Determination of l-(+)-ascorbic acid

L-(+)-ascorbic acid (AA) content was determined by titration with 2,6-dichlorophenolindophenol reagent (AOAC, 2005). Four grams of fruit bar was mixed with 25 ml of an extraction solution [metaphosphoric acid (3% m/v) and acetic acid (8% v/v)]. The mixture was homogenized and centrifuged (model 5430 R, Eppendorf, Germany) at 15,600 rpm and 4°C for 10 min. Ten ml of the supernatant were transferred

to a beaker and titrated with a 2,6 dichlorophenolindophenol solution (0.01%) until a pink color persisted (15 s). The measurements were performed in triplicate and the results were expressed as mg of L-ascorbic acid in 100g of fruit bar.

Total soluble phenolics and antioxidant capacity

Total soluble phenolics and antioxidant capacity was determined following the procedure described in Sedej et al. (2016). Total soluble phenolic (TSP) analysis was adapted from Swain & Hillis (1959). Antioxidant capacity (AOX) assay was based on the method of Brand-Williams, Cuvelier & Berset (1995).

Determination of Color

The color of the fruit bars was determined with a colorimeter (CM508D spectrophotometer, Konica-Minolta Inc., Ramsey, NJ, USA) equipped with D65 at 10° position of the standard observer. CIELAB, color space was applied to perform color measurements. Total color differences between samples and at different storage times were evaluated using the ΔE* values calculated with the following equation (Hirschler, 2012):

$$\Delta E^* = \sqrt{\left(\Delta L^*\right)^2 + \left(\Delta a^*\right)^2 + \left(\Delta b^*\right)^2}$$

where L* value expresses the lightness or luminosity of color, which varies from 0 (absolute black) to 100 (absolute white); a* is associated with changes in redness-greenness (positive a* is red and negative a* is green); and b* is associated with changes in yellowness-blueness (positive b* is yellow and negative b* is blue).

Texture Analysis

Texture studies were performed using a TA-XTPlus Texture Analyzer (Stable Micro Systems Ltd., Godalming, UK). A TA-52 6-mm stainless cylinder probe was used at speed of 1.0 mm s^{-1} and travel distance of 10 mm to puncture the fruit bars. Six replicates were analyzed for each coating. Firmness (g) was measured as the maximum force recorded in the force–time curve during compression.

Microbiological Analyses

The fruit bars were homogenized with Tryptone salt (Biokar diagnostics) in a 1:9 ratio using a stomacher 400 lab blender (Seward, Bohemia, NY). Further decimal dilutions were made with the same diluent. To count yeast and molds, 0.1 ml of each subsequent dilution was spread on potato dextrose agar (Oxoid, Basingstoke, UK) supplemented with 100 ppm chloramphenicol for controlling bacterial growth. The plates were incubated at 25 °C for 5 days and the number of colony-forming units (CFU) per gram of fruit bar was calculated. The microbiological tests were performed in triplicate.

Statistical analysis

Data are presented as mean value ± standard deviation. Analysis of variance was done for all treatments, and the significance of differences between treatments was assessed using Tukey's multiple sample comparison tests. Significance levels were tested at $P \leq 0.05$. All analyses were carried out using Minitab (Minitab Inc., State College, PA, USA) software.

RESULTS AND DISCUSSION

Coating thickness

Fig. 1 shows a representative picture of a coated fruit bar. The coatings appeared smooth with an even surface and there was a clear interface between the bar matrix and coating. The coating adhered well to the bars' surface and even appeared to be absorbed in the pores present at the bar interface. The thicknesses of the different coatings are listed in table 1. No significant differences were observed for the coating thicknesses even though the coating solutions had different viscosity values (Fig 2). Also, the bi-layer coatings had comparable thicknesses to single layer coatings even though the use of calcium is expected to promote a higher number of electrostatic interactions between pairs of carboxylate groups, giving rise to a more viscous solution. All coating solutions had high enough viscosity values that enabled them to be applied as coatings on fruit bars.

Water activity (a_w) and water content

The initial a_w and water content of the fresh fruit bars were 0.610±0.002 and 18.2 % ± 0.1 respectively. The a_w and water content of the coated fruit bars as a function of storage time are shown in figures 4 and 5, respectively. The water activity and water content for all coated samples were approximately the same because all samples were dried to the desired a_w and moisture content of 0.60 and 18%, respectively. The a_w and water content of the fruit bars increased in value over time. The a_w of bars with monolayer coatings was significantly higher than those with bi-layer coatings. In fact, the bars with bi-layer coatings had similar a_w values to those of non-coated bars. The water content values showed a similar trend. The non-coated, Alg-Ch LbL and Alg-Fung Chit LbL coated bars showed no significant differences in moisture content values. These results indicated that the bi-layer coatings were less hydrophilic than the monolayer coatings. Also coatings from different chitosan sources had similar hydrophilic behavior.

L-(+)-ascorbic acid content

Fig.5 shows the retention of L-(+)-ascorbic acid (AA) content during storage. All samples showed a decrease in AA content over time. The coated bars lost between 12 and 40% of AA immediately after coating. This reduction might be due to AA degradation by oxidation during the drying treatment. However, no significant differences in AA content existed between coated and non-coated bars after one week of storage. Furthermore, bars with bi-layer coatings showed the highest ($p<0.05$) AA retention after three weeks, with Alg-Ch LbL and Alg-Fung Ch LbL samples retaining 40% and 45% of AA, respectively. These values were 8 % to 13% higher than that of uncoated bars. In comparison, bars with monolayer coatings showed lower AA retention values. This was due to their higher water contents (Fig. 4) and subsequent increases in AA degradation reaction rates by hydrolysis (De'Nobili, Curto, Delfino, Soria, Fissore & Rojas, 2013). These results indicated a protective effect of the bi-layer coatings on the bars. The retention of AA using by-layer chitosan coatings agreed with the results of Treviño-Garza, García, Herediab, Alanís-Guzmánc & Arévalo-Niñoa, (2017), who found that chitosan and aloe mucilage when applied using the layer-by-layer technique increased AA retention in fresh-cut pineapples.

Total phenolics

Phenolic compounds did not degrade over time for any of the systems studied (Fig 6). The total phenolic content remained constant at 62.2 ± 7.6 mg GAE/g fruit bar (d.b.). These results indicated that the polyphenol oxidase activity might have been inactivated during the production of white grape concentrate as well as during the production of powdered pear flakes.

Antioxidant capacity

Antioxidant capacity showed a steady decrease in value with storage time (Fig 7). No significant differences were observed between different samples, though samples with bi-layer coatings had higher values. Phenolic compounds and ascorbic acid are the major components of fruit bars responsible for antioxidant capacity. Therefore, the reduction in the antioxidant capacity values could be attributed to the decrease in AA content (Fig 5) since the total phenolic content remained constant (Fig 6). The higher values of antioxidant capacity for samples with bi-layer alginate-chitosan coatings correlate with their lower AA loss.

Color evaluation

The color of fruit bars is an important parameter for consumer preference. The color values of the freshly prepared bars were L*=42.0 ± 0.9, a*=11.9 ± 0.7 and b*=24.5±1.5. Figure 8 shows the evolution of L*, a*, b*and ΔE* attributes of the fruit bars during storage. All the bars had positive a* value and appeared reddish. The b* values were also positive, so the bars appeared yellowish. Positive a* and b* values provide an orange hue. Also, the non-coated samples were significantly ($p<0.05$) lighter (L*) and more yellowish (b*) than the coated bars. Coated bars were subjected to air drying, which could have caused AA degradation (Fig 5) and promoted browning of the samples (de Escalada Pla, Delbon, Rojas & Gerschenson, 2009). Also, Maillard reaction during drying might have further contributed to browning of the bars. After 7 days of storage, no significant differences in color were observed between non-coated and coated bars or between bars with different types of coatings. A sharp decrease in L*, a*, b* values occurred between the first and second weeks of storage and these values remained

constant until the end of the experiment. This might have been due to degradation of ascorbic acid, which caused non-enzymatic browning. The first step in degradation of ascorbic acid is part of the non-enzymatic browning reactions chain (León & Rojas, 2007). Irreversible degradation of AA occurs through hydrolysis, simultaneously or competitively with AA oxidation when oxygen is present. The hydrolysis produces 2-keto-L gulonic acid, which becomes involved in dehydration and decarboxylation reactions to produce different browning active compounds (De'Nobili, Curto, Delfino, Soria, Fissore & Rojas, 2013; Pérez, Flores, Marangoni, Gerschenson, & Rojas, 2009). Another parameter that indicates color changes is ΔE*, which is shown in Fig. 8d for all samples as a function of storage time. Differences in perceivable color can be classified as not noticeable (0–0.5), slightly noticeable (0.5–1.5), noticeable (1.5–3.0), well visible (3.0–6.0) and great visible (6.0–12.0) (Cserhalmi, Sass-Kiss, Toth-Markus, & Lechner, 2006). According to this classification, the color changes due to the coatings are considered well visible, whereas the color differences after 7 days of storage are considered great visible. These results suggested that the coatings did not prevent browning of the fruit bars.

Texture evaluation

Immediately after the coating application (time=0), all coated samples showed higher firmness values than those from non-coated bars (Fig 9). The drying treatment during the coating process might have resulted in retracted matrices with components closer to each other, resulting in stronger bar matrices. Also, the coatings might have provided a protective layer that increased the overall firmness of the bar. During storage, the bars became softer due to their higher water contents over time (Fig 4). The Alg-Ch LbL coated bar showed the highest firmness values over the entire storage time, whereas the Alg-fung Ch LbL coated bars showed similar firmness values to the non-coated bars. In comparison, the monolayer coated bars were less firm than the non-coated bars, with the fungal Ch coated bar being the softest one. These results correlated with the water content values of the different samples over time (Fig 4). Previous studies also reported greater firmness of fruits such as mango, strawberries and peaches when coated with a chitosan solution (El Ghaouth, Ponnampalam & Boulet, 1991; Li and Yu, 2000; Zhu,

Wang, Cao, & Jiang, 2008). However, the chitosan concentration in the solution greatly affected the samples' firmness; fruit softening was reduced as chitosan concentration increased from 0.5% to 2% (Bautista-Baños, Hernández-López, Bosquez-Molina & Wilson, 2003; Ali, Muhammad & Siddiqui , 2011).

Antimicrobial Effect of the Applied Coatings

Five fruit bars for each treatment were submitted to microbiological analysis throughout 45 days of storage at room temperature and 100% RH. Table 2 shows the number of bars for each treatment where visible fungal growth was detected.

Fungi and yeast started growing after 28 days of storage in the non-coated, Alg coated and fungal Ch coated bars. The Ch and Alg-Ch LbL coatings delayed yeast and mold growth until day 31, whereas the Alg-fung Ch LbL coating delayed their growth until day 34. Chitosan had widely been used to provide an edible and antimicrobial coating by dipping and spraying of food products (Dutta, Tripathi, Mehrotra & Dutta, 2009). Tamer and Çopur (2010) applied a chitosan-based coating on the surface of strawberries, carrots, mangos, cantaloupes, pineapples and mushrooms and reported that it inhibited microorganism growth as well as improved stability.

CONCLUSIONS

Layer-by-layer chitosan based coatings helped to preserve the quality and increase the shelf-life of fruit bars enriched with ascorbic acid by delaying ascorbic acid degradation and antioxidant capacity loss as well as reducing fruit bar softening. Microbiological analyses also showed a delay in yeasts and fungal growth from 28 days for non-coated fruit bars to 31 days for Alg-Ch LbL coated bars and 34 days for Alg-fung Ch LbL coated bars. Bi-layer coatings had no effect on the a_w, water content and total phenolic content of the bars. In addition, none of the coatings were effective in preventing the browning of the fruit bars.

Acknowledgements

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Authors' contribution

C. Bilbao-Sainz analyzed and interpreted the results. B-S Chiou drafted the manuscript. K. Punotai performed experiments. D. Olson produced the fruit bars. T. Williams and D. Wood performed microscopy analysis. V. Rodov and E. Poverenov extracted the chitosan from mushrooms. T. McHugh designed the study

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Table 1 Coating thickness data

	thickness (μm)
Alg	575.3 ± 199.1
Ch	651.4 ± 215.7
Fung Ch	629.5 ± 212.0
Alg-Ch LbL	501.4 ± 125.5
Alg-Fung Ch LbL	679.5 ± 228.0

Table 2: Number of fruit bars showing visible fungal growth

	Storage time (days)					
	28	31	34	37	41	45
Non-coated	1	3	3	4	5	5
Alg	1	3	4	5	5	5
Ch	0	2	4	4	5	5
Fung Ch	1	2	3	5	5	5
Alg-Ch LbL	0	1	3	4	4	5
Alg-Fung Ch LbL	0	0	2	4	4	5

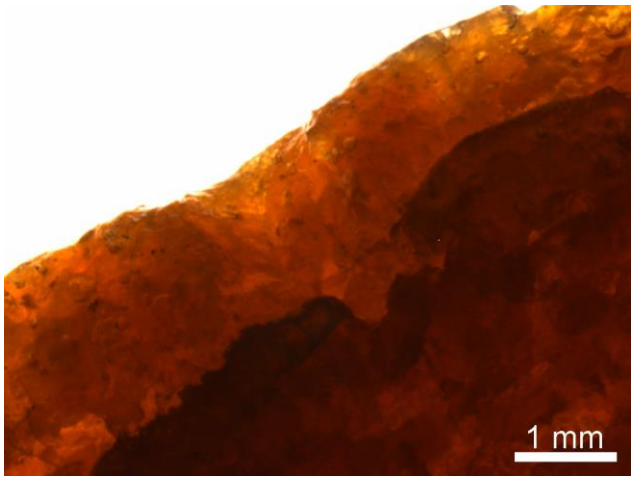

Fig 1 Light micrograph of a fruit bar coated with low molecular weight chitosan

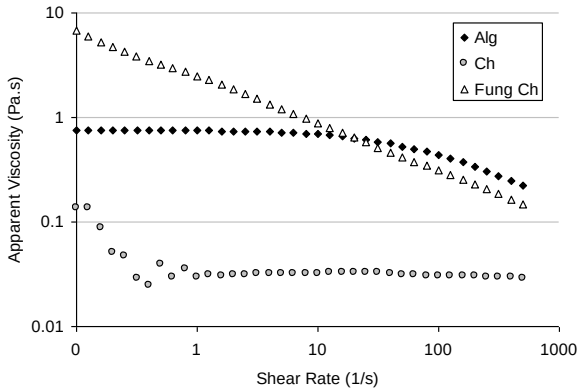

Fig 2 Apparent viscosity-shear rate rheogram for the different coating materials

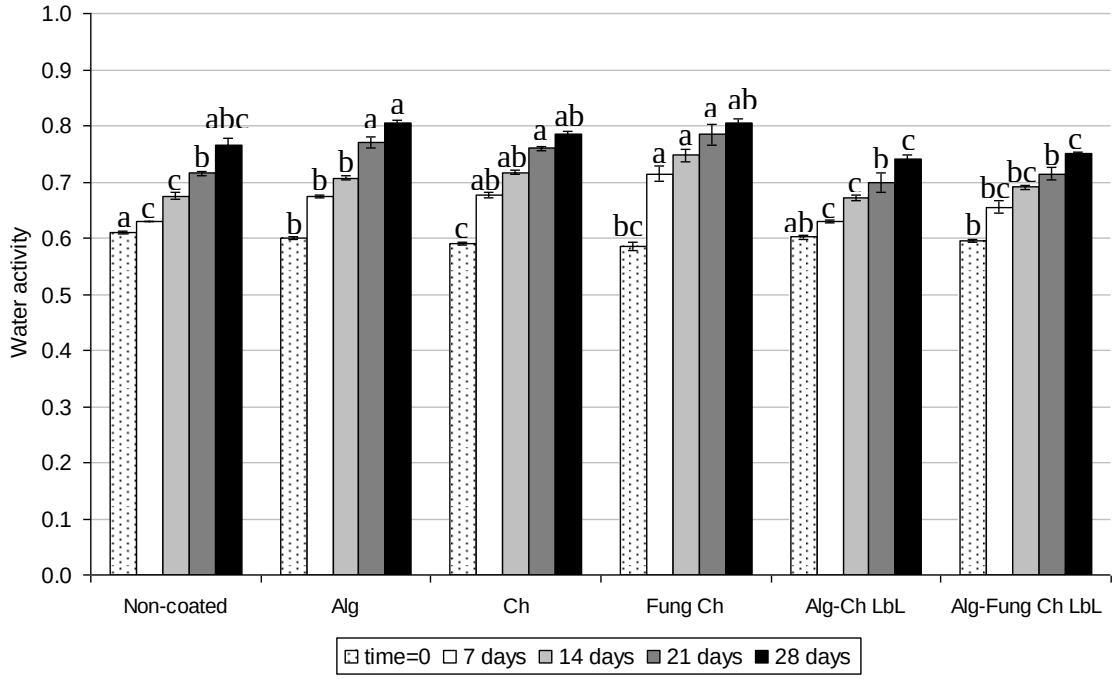

Fig 3 Effect of chitosan, alginate, and alginate–chitosan LbL coatings on water content of fruit bars stored at room temperature and 100% RH. The letters represent comparisons between the treatments at the same storage time. The values followed by the same letter are not significantly different ($p \leq 0.05$)

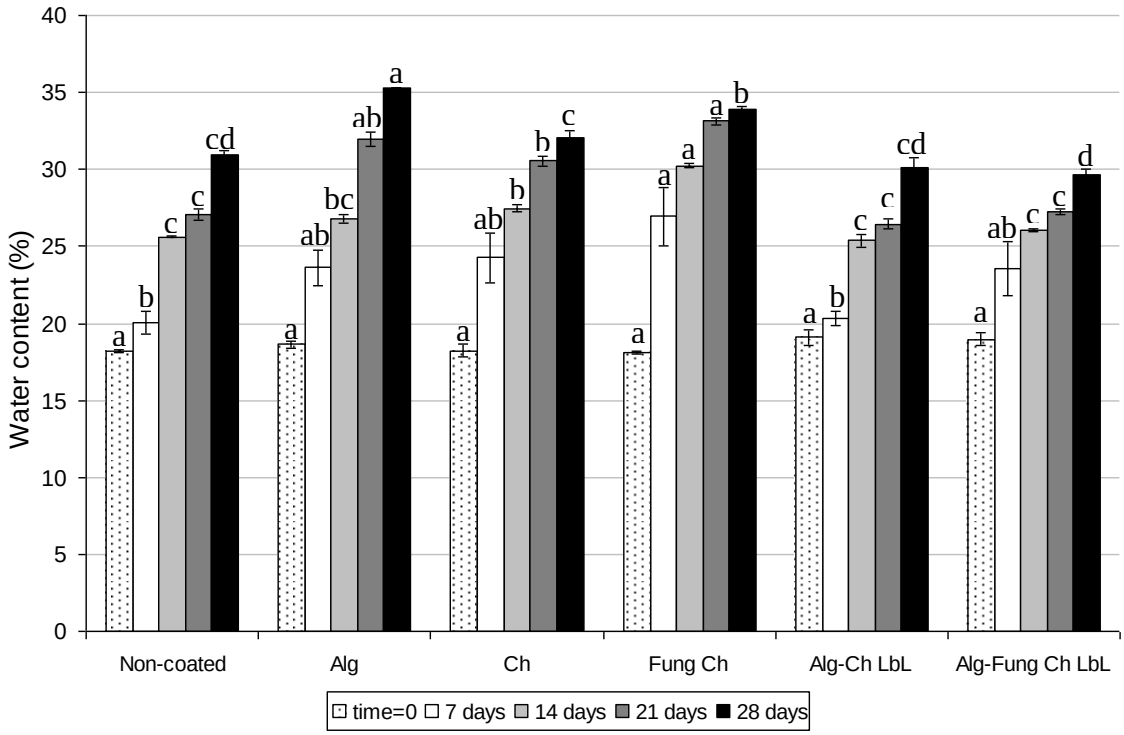

Fig 4 Effect of chitosan, alginate, and alginate–chitosan LbL coatings on water content of fruit bars stored at room temperature and 100% RH. The letters represent comparisons between the treatments at the same storage time. The values followed by the same letter are not significantly different ($p \leq 0.05$)

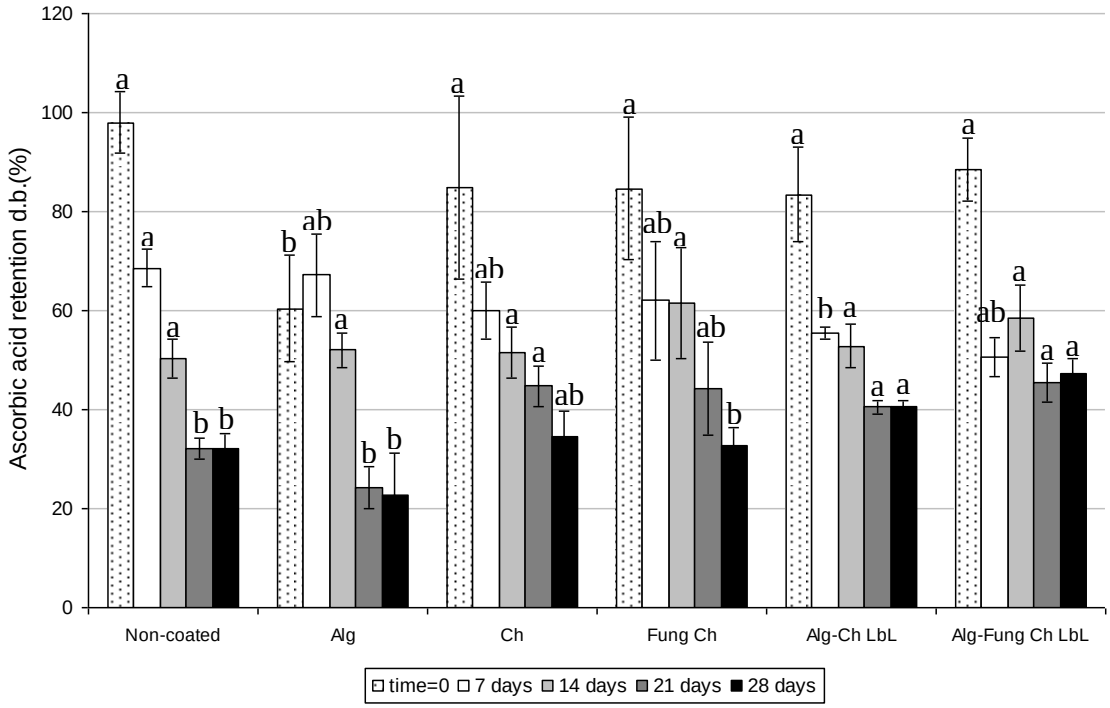

Fig 5 Effect of chitosan, alginate, and alginate–chitosan LbL coatings on ascorbic acid retention of fruit bars stored at room temperature and 100% RH. The letters represent comparisons between the treatments at the same storage time. The values followed by the same letter are not significantly different ($p \leq 0.05$)

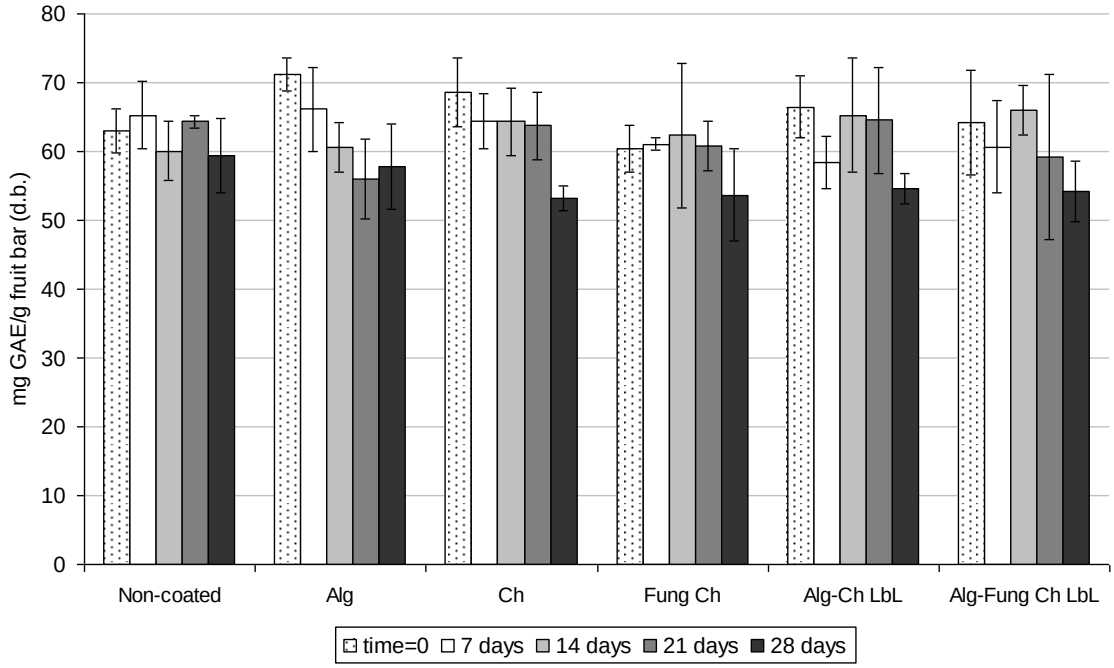

Fig 6 Effect of chitosan, alginate, and alginate–chitosan LbL coatings on total phenolic content in fruit bars stored at room temperature and 100% RH

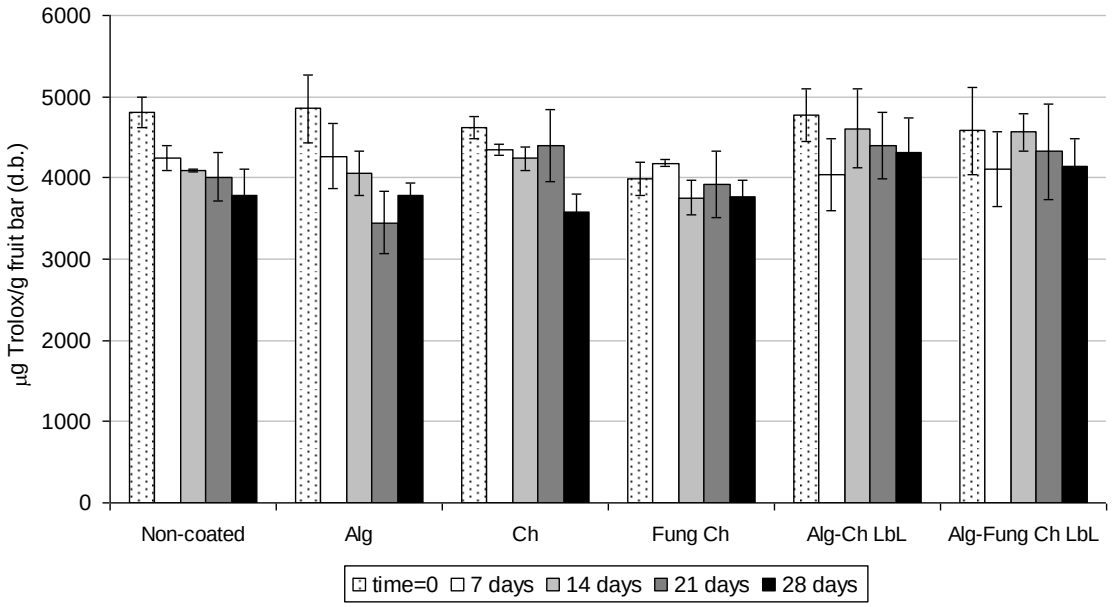

Fig 7 Effect of chitosan, alginate, and alginate–chitosan LbL coatings on the antioxidant capacity of fruit bars stored at room temperature and 100% RH

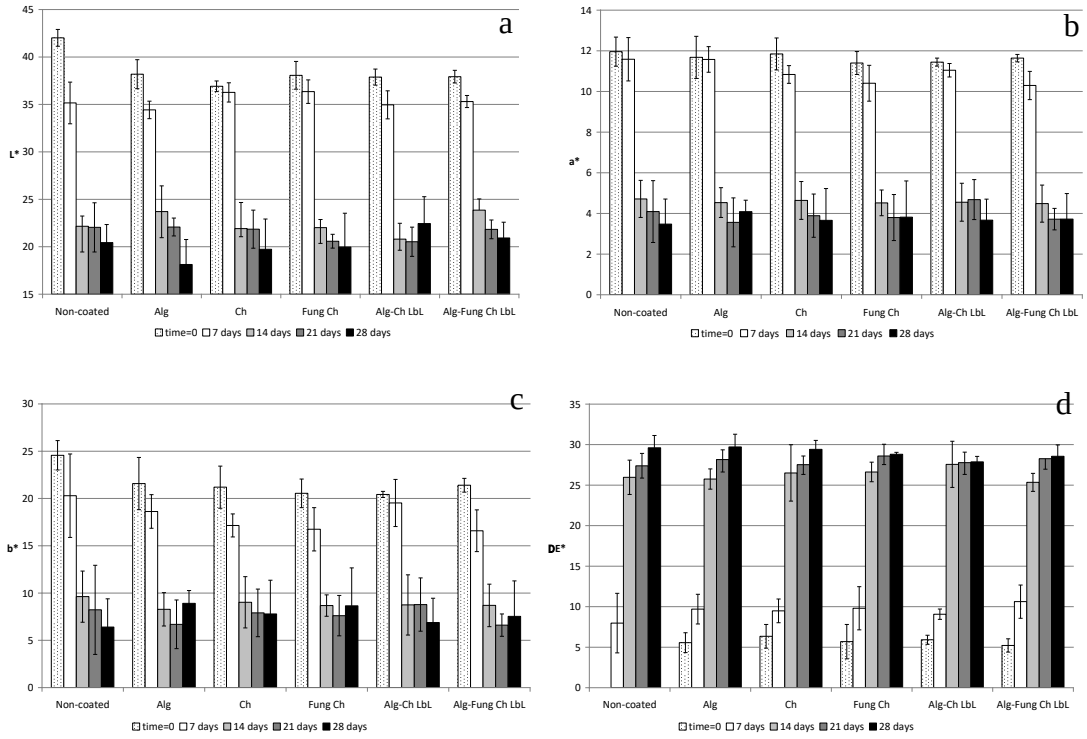

Fig 8 Effect of chitosan, alginate, and alginate–chitosan LbL coatings on CIELab and ΔE* values of fruit bars stored at room temperature and 100% RH

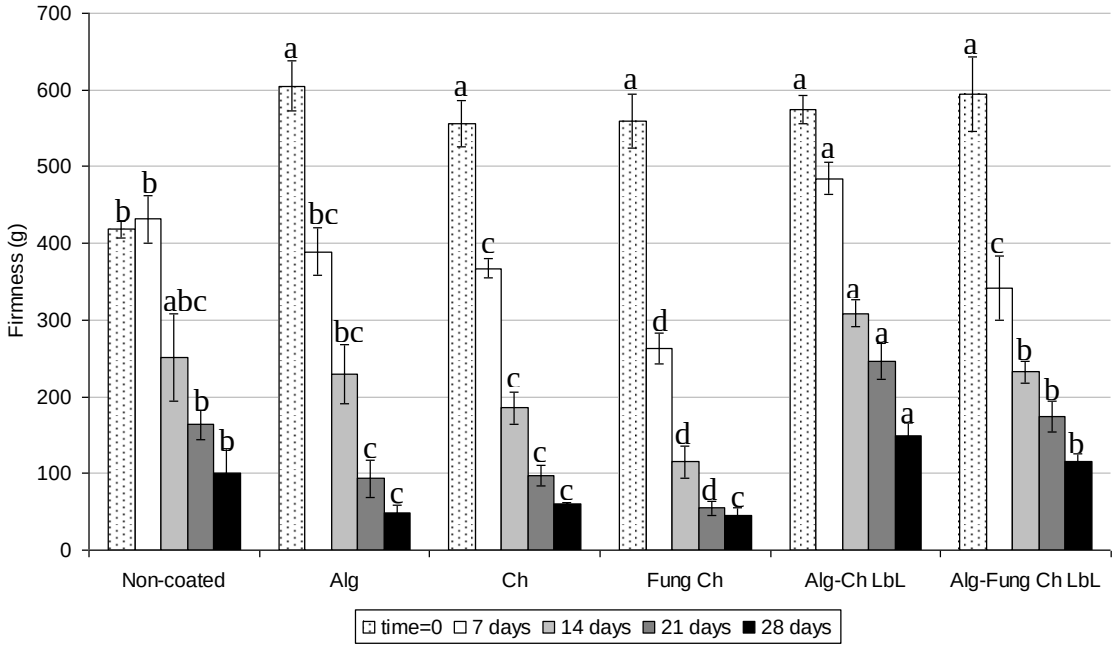

Fig 9 Effect of chitosan, alginate, and alginate–chitosan LbL coatings on fruit bars firmness stored at room temperature and 100% RH. The letters represent comparisons between the treatments at the same storage time. The values followed by the same letter are not significantly different ($p \leq 0.05$)

Paper 5

In preparation, will be submitted to Journal of Food Composition and Analysis

Simplified fast method for the analysis of vitamin D_2 in UV-treated mushrooms

Yakov Vinokur[a], Tara McHugh[b], Cristina Bilbao-Sainz [b], Elena Poverenov[a], Victor Rodov[a,*]

[a] Institute of Postharvest and Food Sciences, Agricultural Research Organization, The Volcani Center, Rishon LeZion 7528809, Israel

[b] Healthy Processed Foods Research, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Albany, CA 94710, USA

* Corresponding author: Dr. Victor Rodov; e-mail: vrodov@ agri.gov.il; Tel: 972-3-9683609; Fax: 972-3-9683622.

ABSTRACT

Mushrooms treated with ultraviolet (UV) light were suggested as a potential dietary source of vitamin D. The existing procedures for vitamin D quantification were developed for fat-rich foods with relatively low vitamin content, e.g. oily fish. In contrast, mushrooms are low in fat and upon UV irradiation accumulate high vitamin D levels in a form of vitamin D_2 (ergocalciferol). A simplified analytical procedure for measuring the content of vitamin D_2 in UV-treated mushrooms has been developed. The procedure omits time- and labor-consuming stages of saponification and preparative enrichment and is based on direct extraction of the vitamin from the ground dried material with subsequent reverse-phase high-performance liquid chromatography (RP-HPLC) analysis. The procedure was found suitable for the analysis of sources containing above 1 µg of vitamin D_2 per gram dry weight, far below the vitamin content reported in UV-treated mushrooms. Irradiation of cut-off stipe bases of common mushrooms (*Agaricus bisporus*) with UV dose of 1.0 J cm^{-2} resulted in vitamin accumulation of about 80 µg g^{-1} for a white strain and about 100 µg g^{-1} for the brown one. The procedure is applicable for time-efficient determination of the vitamin D_2 content in UV-treated mushrooms, e.g. for routine product quality control.

1. Introduction

Mushrooms treated with ultraviolet (UV) light have been suggested as a potential dietary source of vitamin D in a form of vitamin D_2, or ergocalciferol (…). UV in the wavelength range of 290 to 330 nm (UV-B) is the most efficient inducer of photochemical conversion of fungal membrane sterol, ergosterol, into vitamin D_2 (). The practice of UV treatment has been adopted by commercial mushroom growers in order to boost the nutritional value of the produce (). Moreover, converting byproducts of mushroom industry into vitamin-enriched functional food ingredients by a brief UV exposure seems an attractive option (). However, there is still a considerable knowledge gap regarding the irradiation methodology, as well as the bioavailability of the product (). In different reports, the vitamin D_2 content in UV-irradiated mushrooms varies from 2.2 () to 741.5 µg g^{-1} dry mass (). Measuring the vitamin D_2 content in UV-treated mushrooms is necessary for technology optimization, process operational control, product standardization and quality assessment. Such applications require accurate, but fast and feasible analytical procedures.

Significant efforts have been invested in the last fifteen years in elaboration and validation of the methodology for vitamin D analysis in foods (). These methods were primarily developed for quantification of vitamin D_3 (cholecalciferol) in nutritional sources, typically fat-rich fish or artificially fortified products, with relatively low content of the vitamin, 0.4–12 µg/100 g (). The protocols recommended include saponification (alkaline hydrolysis) in order to remove triacylglycerol fats, extraction of non-saponifiable lipids with a non-polar solvent, evaporating this solvent with further reconstitution of the analyte in a mobile phase for reverse-phase high performance liquid chromatography (RP-HPLC), and performing the RP-HPLC analysis, typically with a photodiode-array (PDA) detection. In order to enrich the sample with the analyte, the RP-HPLC stage is often preceded by a preparative-scale, normal phase HPLC separation (). Such procedure is tedious, labor- and time-consuming, prone to losses due to aggressive conditions and analyte transfers, and demands highly skillful operators.

The procedures described above are typically employed also for the analysis of vitamin D_2 in UV-treated mushrooms (), even though the latter material significantly differs of the foods these methods were originally developed for. Mushrooms are low in

fat. Upon UV irradiation, their vitamin D_2 content reaches level 1-4 orders of magnitude higher than vitamin D_3 content in oily fish (the question of functional bioequivalence of these two vitamin forms is out of the scope of the present paper). The vitamin D_2 is not distributed evenly throughout the matrix of the UV-treated mushrooms but concentrated in their superficial layers due to the limited penetration of the UV light. These peculiarities may allow a significant simplification of the vitamin D_2 analysis in UV-treated mushrooms. Thus, the objective of this work was to elaborate a simple procedure applicable for time-efficient determination of the vitamin D_2 content in UV-treated mushrooms, e.g. for routine product quality control.

2. Material and methods

2.1. Material preparation

The mushroom material was prepared as described previously (Bilbao-Sainz et. al., 2017). The cut-off stipe bases of common mushroom (*Agaricus bisporus*) were collected at Monterey Mushrooms, Inc. facility (Watsonville, CA, USA). The stipe batches of white and brown mushroom strains were collected separately. The material was stored at 5°C overnight and subsequently washed and cut transversely into slices of 6-8 mm thick, typically 2 to 3 cm in diameter. The slices were placed flat onto a conveyor of the Uvitron UV Conveyor 40 dual-lamp curing system equipped with two SunRay 400 SM UVB flood lamps (Uvitron International Inc., West Springfield, MA, USA) and subjected to UV-B treatment. UV-B dose (energy) and peak intensity (power) were measured using an Uvicure Plus II radiometer (EIT Inc., Sterling, VA, USA). The lamp height was set at 3.8 cm to achieve a peak intensity of 492 W/m^2. The exposure duration was varied by adjusting the conveyor speed to achieve the UV-B doses of 0.25, 0.5 and 1.0 J cm^{-2}. After passing the system the slices were turned upside down and the same UV-B dose was applied to the opposite side of the slice. The control samples were passed through the system with the lamps turned off. After the treatment, the mushroom slices were freeze dried in a Labconco Freezone 12 Freeze Dry System (Kansas city, MO, USA), sealed under nitrogen in laminated PET bags (Impak corp., Los Angeles, CA, USA) and sent to the ARO (Israel) with a courier service for further analysis. At the ARO, the material was stored in the original sealed packages at -18°C until the analysis. Prior to analysis, the slices of uniform thickness of ca. 6 mm were selected, and discs of 17 mm in diameter

were excised from them with a cork borer, in order to standardize the irradiated surface area. If not specified differently in the text, the discs were ground to a fine powder with particle sizes smaller than 0.06 mm.

2.2. Vitamin D extraction

Vitamin D_2 was extracted from the mushroom material by two procedures, either a standard process based on the official AOAC 2002.05 method (AOAC, 2005) with modifications, or a proposed simplified process. The standard procedure included alkaline hydrolysis (saponification) with subsequent extraction of the hydrolysate with n-heptane. The major modification of the method was smaller analyte amount taken and proportional downscaling of the whole procedure due to the higher content of the vitamin D in the UV-treated mushrooms as compared with the foods the method was originally intended for. The saponification medium comprised 60 mL of absolute ethanol, 10 mL of ascorbic acid solution 5% w/v, and 10 mL of aqueous 50% w/v potassium hydroxide. The ground mushroom material (100 mg) was spiked with 50 µL of ethanol containing 5 µg of vitamin D_3 (internal standard), and further suspended in 4.95 mL of the saponification medium, to reach the final vitamin D_3 concentration of 1 µg mL^{-1}. For hydrolysis, the samples were kept on a water bath at 95°C for 30 min.

After the hydrolysis, 2 mL of water was added to the sample, it was cooled down to room temperature with subsequent addition of 5 mL 40% v/v ethanol and mixing. The obtained sample was extracted with three portions of 3 mL n-heptane. The heptane extracts were combined and washed sequentially with 2 mL 1 M KOH, 5 mL 40% v/v ethanol (twice) and 5 mL deionized water (at least twice). The washing procedure was performed until the heptane phase became free of alkali as detected by phenolphthalein solution. One granule of BHT was added to the extract, and the heptane was evaporated to dryness on a rotary evaporator at 45°C. The residue was reconstituted in 5 mL of the HPLC mobile phase acetonitrile-methanol (3:1, v/v). At this stage, the extract could be stored at 4°C overnight until the HPLC analysis.

With the simplified method, the ground mushroom material (100 µg) was spiked with 50 µL of the acetonitrile-methanol mobile phase (see above) containing 10 µg of vitamin D_3 (internal standard), and further suspended in 2.95 mL of the mobile phase. The suspension was vortexed for 2 min and separated by centrifugation (5,000 rpm, 3 min).

The extraction was repeated with two additional 3-mL portions of the mobile phase. The supernatants were combined and brought with the same solvent mix to the total volume of 10 mL, to reach the final internal standard concentration of 1 μg mL^{-1}. The extract could be stored at 4°C overnight until the HPLC analysis. In one trial, the same procedure was performed with unground mushroom discs material.

The extraction recovery rate for the both methods was determined by quantification of the spiked vitamin D_3 using an external standard calibration curve.

2.3. HPLC analysis

The HPLC analysis was performed using a Prominence HPLC system (Shimadzu, Kyoto, Japan) with an Inertsil ODS-P C-18 RP column (GL Sciences, Japan), 250 ×4.9 mm I.D., and particle size 5 μm, flow rate 1 mL/min, injection volume 50 μL, column temperature 40°C, eluents: A (75% acetonitrile, 25% methanol) and B (100% ethanol). The gradient program was as follows: 0 to 11 min, linear gradient from 100% to 90% eluent A; 11 to 15 min, linear gradient from 90% to 20% eluent A; 15 to 19 min, linear gradient to 100% eluent B; 19 to 24 min, 100% eluent B; 24 to 32 min, linear gradient back to 100% eluent A. The detection was conducted using a SPD-M20A PDA detector at 264 nm. The vitamin D_2 peak was identified by comparison with authentic sample (Acros Organics, USA) and quantified using an internal standard of vitamin D_3 (Acros Organics, USA).

2.4. LC-MS identification

The identity of the vitamin D_2 in the extracts prepared by the simplified method was checked by LC-MS analysis in comparison with authentic standard on an LC-MS system which consisted of Dionex Ultimate 3000 RS HPLC coupled to Q Exactive Plus hybrid FT mass spectrometer equipped with atmospheric pressure chemical ionization (APCI) source (Thermo Fisher Scientific Inc., Waltham, MA, USA). Chromatographic separations were carried out according to the HPLC method presented above except for using methanol-acetic acid 0.1% (97:3, v/v) instead of acetonitrile-methanol (3:1, v/v) as eluent A. Mass spectrometer was operated in positive ionization mode, ion source parameters were as follows: corona discharge needle current 5μA, capillary temperature 300°C, sheath gas rate (arbitrary units) 50, and auxiliary gas rate (arbitrary units) 10. Mass spectra were acquired in scan mode; resolving power was 70.000. The LC-MS

system was controlled and data were analyzed using Xcalibur software (Thermo Fisher Scientific Inc).

2.5. Statistics

The analyses were performed at least in triplicate. The Microsoft Office Excel spreadsheet was used to calculate means, standard deviations, and 95% *t* confidence intervals. The statistical analyses were carried out using JMP version 5.0.1 software (SAS Institute Inc., Cary, NC) including a one-way analysis of variance (ANOVA) followed by the Tukey's 5% honestly significant difference (HSD) post hoc test.

3. Results and discussion

The LC-MS analysis of the extract prepared by the simplified procedure from UV-treated mushrooms showed that its major UV-absorbing peak was identical to the authentic ergocalciferol, both by retention time and by mass spectrum, having the characteristic $[MH]^+$ ions at m/z 397.3 and 379.3 (Fig. 1). In addition, the purity of the putative vitamin D_2 peak was confirmed by the PDA HPLC analysis. These findings confirmed that the results of the simplified analytical HPLC procedure indeed reflected the vitamin D_2 content in the samples. Minimal vitamin concentration measurable by this procedure was about 1 μg g^{-1}, well below the least vitamin D_2 content reported in UV-treated mushrooms.

No significant difference was found in the efficacy of the vitamin D_2 extraction by the simplified procedure and by the standard method including saponification (Table 1). As expected, extraction of the excised superficial layers (approximately 1.5 mm from each side of the 6-mm thick disk) showed twice as high vitamin content per weight unit as the whole disk. In the other words, practically all ergocalciferol was concentrated in these superficial layers while the inner tissues contained no vitamin. Not surprisingly, UV irradiation of thin mushroom slices was reported to increase significantly the vitamin output as compared with whole mushrooms treatment (). Even though the vitamin was concentrated in the superficial layers, the attempts of extracting unground discs showed low efficacy (Table 1). Grinding the dried mushroom tissues prior to extraction was necessary for exhaustive elution of the vitamin. In that case, practically no vitamin was left in the residue after the extraction (data not shown).

The amount of vitamin in non-irradiated mushrooms was not detectable by the simplified procedure. Increasing the UV-B dose from 0.25 to 1.0 J cm^{-2} resulted in significant increase of the vitamin content reaching about 80 µg g^{-1} for white mushrooms and about 100 µg g^{-1} for the brown ones (Fig. 2). In a separate trial, mushrooms irradiated with UV-B doses of 3.0 to 5.0 J cm^{-2} were analyzed showing the maximal vitamin contents of 109.4 ± 9.1 for white and 148.2 ± 6.2 for brown mushrooms. The trend of the brown *Agaricus* mushrooms to respond to UV irradiation with somewhat higher vitamin D_2 accumulation than in the white mushrooms has been mentioned in the literature ().

4. Conclusions

A simplified analytical procedure for measuring the content of vitamin D_2 in UV-treated mushrooms has been developed. The procedure omits time- and labor-consuming stages of saponification and preparative enrichment and is based on direct extraction of the analyte from the ground dried material with subsequent RP-HPLC analysis. The procedure was found suitable for the analysis of sources containing above 1 µg of vitamin D_2 per gram dry weight. Irradiation of cut-off stipe bases of common mushrooms (*Agaricus bisporus*) with UV dose of 1.0 J cm^{-2} resulted in vitamin accumulation of about 80 µg g^{-1} for a white strain and about 100 µg g^{-1} for the brown one. The procedure is applicable for time-efficient determination of the vitamin D_2 content in UV-treated mushrooms, e.g. for routine product quality control.

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References

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Table 1. Effect of material preparation and extraction methods on vitamin D_2 yield from white mushroom stipe cut-offs treated with UV-B in a dose of 1.0 J cm^{-2} (recovery percentage in brackets)

	Material preparation		
Method	Ground	Unground	Surface layer, ground
Standard (saponification)	76.4 ± 4.3 b (99.4%)	21.1 ± 3.0 a (61.4%)	n.d.*
New (direct extraction)	78.0 ± 2.2 b (99.6%)	29.7 ± 5.4 a (68.5%)	161.6 ± 11.7 c (100.3%)

Values represent means of three replications ± 95% *t* confidence intervals. The means were separated by Tukey's 5% honestly significant difference (HSD) post hoc test. The values followed by the same letter are not significantly different at $p \leq 0.05$; *n.d. – not determined.

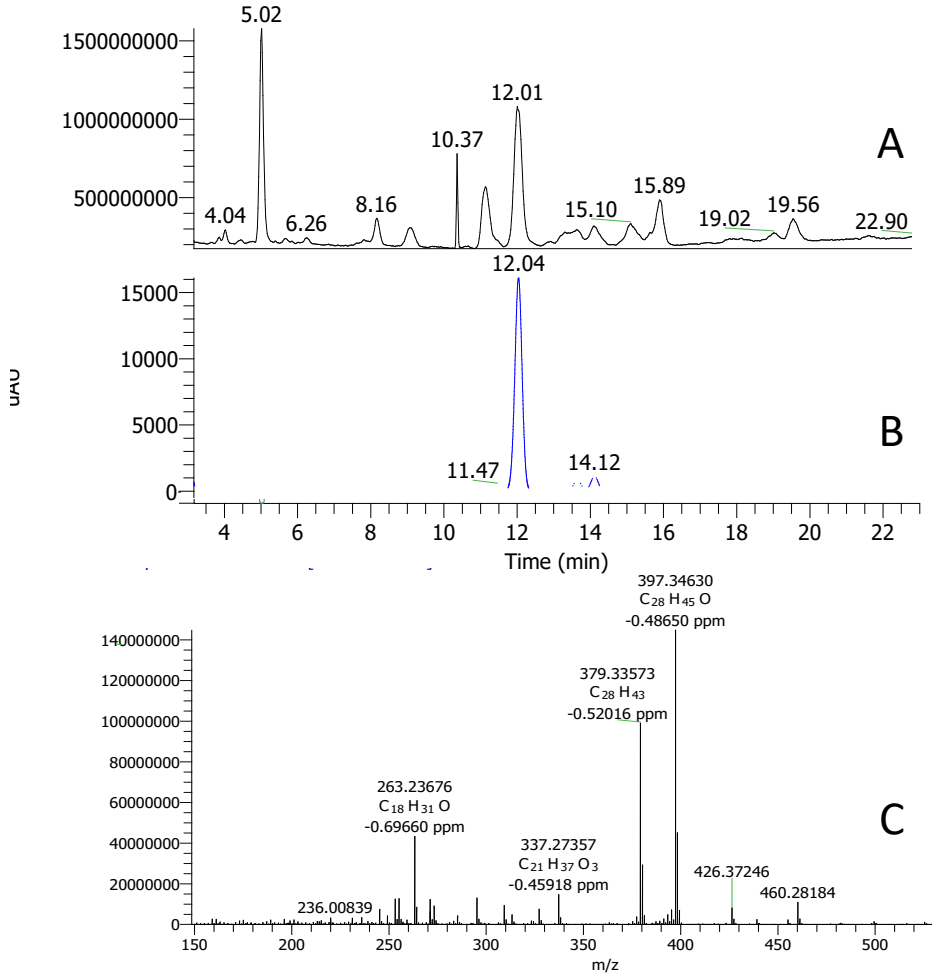

Figure 1. Positive APCI total ion current (A) and UV (B) chromatograms of the UV-B irradiated mushroom sample; positive APCI mass spectrum of vitamin D_2 detected in the sample at retention time 12.01 min.

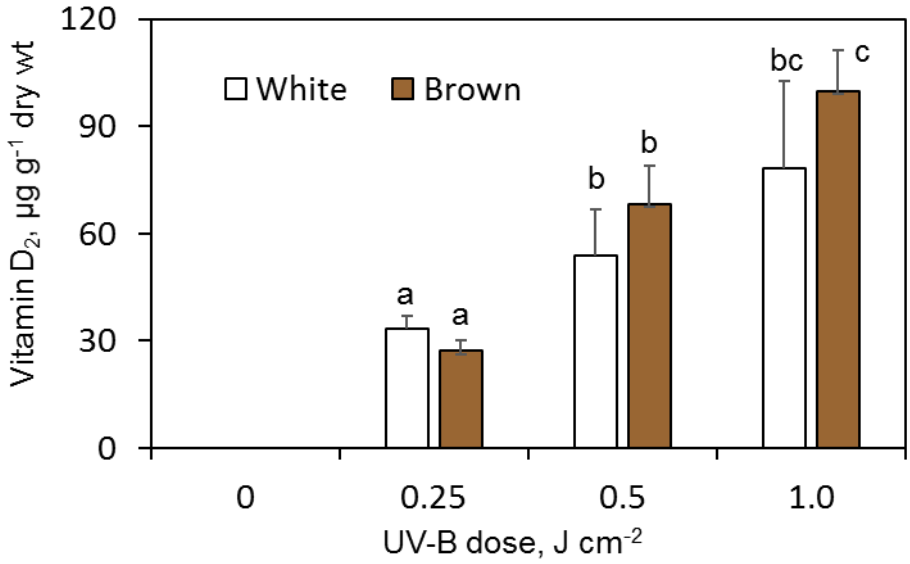

Figure 2. Effect of UV-B dose on the content of vitamin D_2 in the stipe cut-offs of white and brown mushrooms. Values represent means of three replications; error bars represent 95% *t* confidence intervals. The means were separated by Tukey's 5% honestly significant difference (HSD) post hoc test. The values followed by the same letter are not significantly different at $p \leq 0.05$